EXCRETION OF THE TWO BIOLOGICALLY ACTIVE FORMS OF ASCORBIC ACID AND OF DIKETOGULONIC ACID BY HUMAN SUBJECTS [1]

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(Received for publication April 24, 1951)

Blood levels and urinary excretion of ascorbic acid have been used as criteria for judging the vitamin C nutrition of individuals and the availability of the vitamin from different sources.

In the earlier studies reduced ascorbic acid only was determined. The method most frequently employed was based upon the indophenol reaction. Later a measure of dehydroascorbic acid as well as reduced ascorbic acid was obtained by preceding the analysis by treatment with H_2S.

Penney and Zilva ('43) reported a study on guinea pig tissues, blood and urine in which they measured reduced and dehydroascorbic acid by titration with indophenol, and diketogulonic acid by its reaction with dinitrophenylhydrazine. An intensely red color was obtained by solution in NaOH of the derivative formed in this reaction.

Roe and Kuether ('43) published a method for determination of total ascorbic acid. This method made use of the reaction betwen 2–4 dinitrophenylhydrazine and dehydroascorbic acid and the development of a red color on the addition of 85% H_2SO_4. Oxidation of reduced ascorbic acid to the dehydro form preceding analysis made possible the determination of total ascorbic acid.

[1] This work was aided by a grant from the Purdue Research Foundation.

[2] Based on material constituting part of a doctoral thesis submitted by this author to the graduate school of Purdue University.

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Berryman et al. ('44), using the indophenol method for reduced ascorbic acid and the dinitrophenylhydrazine method of Roe and Kuether for total ascorbic acid, and computing the dehydroascorbic acid by difference, obtained results indicating the presence of small quantities of dehydroascorbic acid in urine.

Penney and Zilva ('45), Guild et al. ('48) and Hartzler ('48) presented evidence that the Roe and Kuether method measured, in addition to dehydroascorbic acid, another derivative, which Penney and Zilva stated was 2, 3-diketogulonic acid.

Roe et al. ('48) reported procedures for using the dinitrophenylhydrazine method for determining diketogulonic, dehydroascorbic and reduced ascorbic acids in the presence of each other. Following the appearance of this report, we became interested in making observations on the urinary excretion of the three substances by human subjects under different dietary regimes and with ascorbic acid intakes from different sources. No previous studies of this nature have, to the knowledge of the present authors, been reported.

EXPERIMENTAL PROCEDURE

The subjects were 6 apparently healthy college women. Two were graduate students who were majoring in biochemistry and nutrition and 4 were undergraduate nutrition majors. The ages of the graduate students were 33 and 35 years respectively, and the undergraduates ranged from 20 to 22 years of age.

Excretion values for diketogulonic acid and dehydro and reduced ascorbic acid were obtained for the two graduate subjects: (1) on their usual diets; (2) on diets very low in fruits and vegetables; (3) on low fruit and vegetable diets supplemented with tablets containing 25, 50, and 75 mg ascorbic acid. The low fruit and vegetable diets were only roughly controlled.

The 4 undergraduate subjects were placed on a constant diet, which was supplemented with: (1) crystalline ascorbic

acid; (2) cantaloupe; and (3) grapefruit. The ascorbic acid-containing foods of the constant diet, the cantaloupe and the grapefruit were analyzed for diketogulonic acid and dehydro and reduced ascorbic acids, using the phenylhydrazine method of Roe et al. ('48). The amount of crystalline ascorbic acid fed as a supplement was 40 mg, and the cantaloupe and grapefruit were fed in amounts to give an approximately equal intake in terms of the two forms of ascorbic acid and diketogulonic acid. The constant diet used is given in table 1.

TABLE 1

Constant diet

BREAKFAST		LUNCH		DINNER	
Grape juice (gm)	120	Cream of pea soup (gm)	200	Meat patties (gm)	100
Milk (gm)	200	American cheese (gm)	40	Mashed potatoes (gm)	150
Rice Krispies (gm)	20	Apple, raw (gm)	50	Green beans, canned (gm)	75
Toast and butter	ad lib.	Dates (gm)	35	Pears, canned (gm)	80
Egg	1 med.	Crackers and cookies	ad lib.	Carrot strips (gm)	20
Cream (gm)	30			Bread, butter and cookies	ad lib.
Coffee	ad lib.				

Analysis of the diet gave average daily values of 8.42 mg diketogulonic acid, 6.16 mg dehydroascorbic acid, 29.67 mg reduced ascorbic acid, and a total for the three substances of 44.25 mg. The average daily values for the cantaloupe supplements for a 6-day feeding period were 11.36 mg diketogulonic acid, 8.72 mg dehydroascorbic acid and 26.57 mg reduced ascorbic acid, with a total of 46.65 mg. The average daily values for the grapefruit supplements, covering the same period of time, were 1.25 mg diketogulonic acid, 0.99 mg dehydroascorbic acid, and 43.94 mg reduced ascorbic acid, with a total of 46.18 mg.

The urine was analyzed by the same method as that employed for the foods. It was preserved during collection with metaphosphoric acid to which a stannous chloride solution

was added as described by Roe et al. ('48) for extraction of animal tissues. This procedure prevents oxidation of reduced ascorbic acid to dehydroascorbic acid and is believed to maintain at a minimum the conversion of the latter to diketogulonic acid. Immediate analysis of a urine sample and analysis after preservation in this way for 24 hours showed little change in the quantities of the three compounds present.

Six-day feeding periods preceded by a 5-day saturation period were used, but results are reported for only the last

TABLE 2

Excretion of the two forms of ascorbic acid and diketogulonic acid on different diets (ave. for three days — mg)

TYPE OF DIET	SUBJECT S			SUBJECT T		
	DKA	DHA	AsA	DKA	DHA	AsA
Customary	3.13	6.22	62.25	4.01	4.00	29.17
Low fruit and vegetable: unsupplemented	2.39	3.41	29.59	3.99	3.41	23.59
Supplemented with AsA tablets:						
25 mg	3.25	6.15	23.17	3.92	3.88	18.06
50 mg	3.08	5.99	23.22	3.05	4.34	18.45
75 mg	3.44	4.40	37.50	3.19	3.58	26.86

4 days, since the first two days usually reflected the high intake of ascorbic acid during the saturation period.

RESULTS

The urinary excretion of the two forms of ascorbic acid and of diketogulonic acid for subjects on customary diets and on low fruit and vegetable diets, unsupplemented and supplemented with crystalline ascorbic acid, are recorded in table 2. Subject S was in the habit of consuming larger amounts of fruits and vegetables than subject T and this was reflected in a much higher excretion of reduced ascorbic acid when

the subjects were on their customary diets. The diketogulonic acid of the urine did not seem to be related to the fruit and vegetable intake; this may not have been true with respect to the dehydroascorbic acid. When both subjects were on low vegetable and fruit diets, there was not a marked difference in excretion values. Supplementation with ascorbic acid tablets

TABLE 3

Intake and excretion of the two forms of ascorbic acid and diketogulonic acid on constant diet plus supplements (ave. 3rd to 6th day — mg)

	DIET PLUS CANTALOUPE		DIET PLUS CRYST. ASC. ACID		DIET PLUS GRAPEFRUIT	
	Intake	Excretion	Intake	Excretion	Intake	Excretion
SUBJECT A [1]						
DKA	19.78	6.39	8.42	5.41		
KHA	14.88	6.55	6.16	8.23		
AsA	56.24	50.99	69.67	40.95		
SUBJECT B						
DKA	19.78	3.30	8.42	3.47	9.67	3.77
DHA	14.88	4.26	6.16	6.48	7.15	5.80
AsA	56.24	30.54	69.67	31.82	73.61	42.88
SUBJECT C						
DKA	19.78	4.93	8.42	6.53	9.67	6.25
DHA	14.88	7.47	6.16	6.49	7.15	5.32
AsA	56.24	51.16	69.67	41.16	73.61	47.56
SUBJECT D						
DKA	19.78	4.25	8.42	4.02	9.67	4.56
DHA	14.88	5.72	6.16	5.72	7.15	5.05
AsA	56.24	36.16	69.67	41.70	73.61	20.18

[1] Subject A was unable to participate during grapefruit period.

at the levels used exerted little influence on the excretion of the three substances. However, there was a slightly higher average output of reduced ascorbic acid at the 75-mg level. This was greater for subject S than subject T.

Table 3 records the average daily intake and excretion of the two forms of ascorbic acid and of diketogulonic acid for subjects on a constant diet with cantaloupe, crystalline ascorbic acid and grapefruit supplements. Average outputs with the

three supplements did not show large variations among the subjects, with the exception of subject D, whose excretion of reduced ascorbic acid during the grapefruit period was markedly decreased. This subject contracted a cold at the beginning of that period and it is thought that this may have increased tissue needs, with a resulting lowered output.

Since the constant diet contained both forms of ascorbic acid as well as diketogulonic acid, all three substances were furnished by the basal diet in like amounts during the three periods, but the quantities of each furnished by the supplement differed. As the crystalline ascorbic acid furnished no dehydroascorbic acid or diketogulonic acid, the intake of these substances with this supplement was not increased above the basal intake. However, the intake of both of the above substances was markedly increased by the addition of cantaloupe to the basal diet, and slightly increased by the addition of grapefruit. This increased intake was not reflected in the excretion. In all cases, except for dehydroascorbic acid with subjects on the crystalline ascorbic acid supplement, the output of dehydroascorbic acid and diketogulonic acid was less than the intake. This difference was marked with the cantaloupe supplement. Existing evidence from other studies indicates that a high percentage of dehydroascorbic is utilized by the tissues but that diketogulonic acid is not biologically active. Penny and Zilva ('43) have suggested that dehydroascorbic acid is partly reduced to ascorbic acid rapidly by the organism and partly converted into diketogulonic acid. According to these investigators, some of the diketogulonic acid may be deposited in the tissues and some excreted in the feces and urine. They also state that the occurrence of dehydroascorbic acid and diketogulonic acid in urine may be due in part to oxidation during filtration and storage in the kidneys and bladder.

The observations made in the course of this study suggest a need for more information on the metabolic relationships among the two forms of ascorbic acid and diketogulonic acid.

The use of isotopic tracers would, no doubt, prove helpful in investigation of this subject.

SUMMARY

The excretion of reduced ascorbic acid, dehydroascorbic acid and diketogulonic acid has been observed with subjects on customary diets; on low vegetable and fruit diets, unsupplemented and supplemented with varying levels of ascorbic acid in the form of tablets; and on a constant basal diet supplemented with crystalline ascorbic acid, cantaloupe and grapefruit.

All three substances were excreted on all dietary regimes. A larger intake of fruits and vegetables resulted in increased excretion of reduced ascorbic acid, but had little or no effect on the output of dehydroascorbic acid and diketogulonic acid.

Supplementation of low vegetable and fruit diets with crystalline ascorbic acid likewise increased the reduced ascorbic acid output when intake was increased from a 25 to a 75 mg level, but had little or no influence on the dehydroascorbic acid and diketogulonic acid output.

The higher intake of dehydroascorbic acid and diketogulonic acid on the constant diet plus the cantaloupe supplement did not increase the excretion of these two substances above that observed on the constant diet supplemented with crystalline ascorbic acid and grapefruit. The average excretion of reduced ascorbic acid for a given subject did not vary greatly for the different supplements.

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NITROGEN BALANCE OF ADULT RATS FED DIETS LOW IN L- AND DL-THREONINE [1]

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ONE FIGURE

(Received for publication May 1, 1951)

Previous investigations of the utilization of threonine have been limited almost wholly to observations of the growing animal. All investigators agree with Rose ('38) that only the naturally occurring isomer is useful in promoting growth. Rose ('49) determined the threonine requirement of the adult human for nitrogen balance, and Benditt et al. ('50) reported similar data for the adult rat. The present investigation was undertaken in order to provide more information concerning the utilization of L- and DL-threonine in the maintenance of nitrogen equilibrium by the adult rat.

METHODS

Two groups of adult male albino rats (series 160 and 240) were used. After a 48-hour fast the body weights of series 160 ranged from 238 to 213 gm (mean, 224 gm); under the same conditions the rats of series 240 ranged in weight from 274 to 214 gm (mean, 245 gm). The caloric allowance for series 160 was 39.4 Cal./rat/day, and for series 240 it was 42.1 Cal./rat/day (Cal./rat/day $= 121\ kg^{3/4}$). Each experiment included the following sequence of feedings: maintenance diet (9.6% whole egg protein), two weeks; nitrogen-free (N-free) diet,

[1] This investigation was supported in part by a grant from the Office of Naval Research, NR contract N6ori-126 Task IX.

one week; amino acid diet, containing approximately half the maintenance requirement of nitrogen (half-N), one week; amino acid diet, containing approximately the maintenance requirement of nitrogen (full-N), one week. This 5-week cycle of feeding was repeated for each amino acid mixture investigated.

TABLE 1

Composition of diet containing "complete" amino acid mixture (Grams per kilogram of diet)

L-Arginine · HCl	3.77	DL-Valine	4.67
L-Histidine · HCl	1.28	L-Glutamic acid	11.12
DL-Isoleucine	4.45	Sucrose	604.0
L-Leucine	3.10	Vitamin mixture[1]	58.2
L-Lysine · HCl	2.82	Salt mixture[2]	45.1
DL-Methionine	2.48	Cellulose flour	53.0
DL-Phenylalanine	3.94	Cottonseed oil	174.6
DL-Threonine	3.20	Fecal marker[3]	23.2
DL-Tryptophan	1.08		

[1] Mixed with sucrose to supply the following per kilogram of diet: 9.2 mg thiamine hydrochloride, 9.2 mg pyridoxine hydrochloride, 18.4 mg riboflavin, 46.0 mg niacin amide, 51.0 mg calcium pantothenate, 0.6 mg biotin, 0.6 mg folic acid, 11.5 mg methyl naphthoquinone, 46.0 mg alpha-tocopherol, 72,800 units vitamin A, 14,560 units of vitamin D, 1.15 gm choline chloride.

[2] Wesson ('32).

[3] SiO_2 or Cr_2O_3.

The composition of the diet containing the "complete" amino acid mixture is given in table 1. This mixture simulates the proteins of whole egg in that it contains approximately the same amount of each natural isomer of the essential amino acids per gram of total nitrogen. The non-essential amino acids of egg protein are replaced, in this mixture, by the D-forms of 6 essentials plus sufficient L-glutamic acid to make up the same amount of total nitrogen (Anderson and Nasset, '48).[2]

[2] Amino acids and vitamins were provided by Merck and Company. Folic acid was provided by the Lederle Laboratories.

The N-free diet was compounded simply by omitting the amino acid mixture shown in table 1 and adding an equal weight of sucrose.

In this laboratory the utilization of a particular essential amino acid is measured when its concentration in the mixture is reduced sufficiently to make it the limiting factor in the utilization of the mixture for the maintenance of nitrogen equilibrium. In the present experiments one-third DL-, one-sixth L-, one-fourth DL-, and one-eighth L-threonine mixtures were used. The fractions indicate the amounts of threonine present, regardless of isomeric form, as compared with the "complete" amino acid mixture shown in table 1. For example, one-eighth L-threonine refers to a mixture which contains only one-eighth as much threonine as the complete mixture.

The N-free, half-N and full-N diets were fed by stomach tube in two equal portions daily. Feces were marked by adding Cr_2O_3 to the first feeding in each period. In order to maintain the same quantity of inert material in the diet, SiO_2 was substituted for Cr_2O_3 during the remainder of the period. The change in color is very sharp and both of these oxides appear to be quite inert. Feces were collected for the entire 7-day period; urine was collected only for the last 4 days. Other details of the experimental procedure were described by Nasset and Anderson ('51).

RESULTS

Table 2 contains the average results of all experiments. The recorded body weight is the average of the three body weights obtained at the end of the N-free, half-N and full-N periods in each experiment. The maximum change between adjacent periods was a gain of 5.1% in experiment III of series 240.

The addition of the complete amino acid mixture to the N-free diet resulted in the usual diminution in urinary nitrogen. The magnitude of the change was the same whether half or full maintenance quantities of nitrogen were fed. This im-

TABLE 2

Average nitrogen balance data for rats receiving amino acid mixture diets

EXPERIMENT NUMBER	SERIES 160			SERIES 240		
	I	II	III	I	II	III
Amino acid mixture	Complete	1/3 DL-Threonine	1/6 L-Threonine	Complete	1/8 L-Threonine	1/4 DL-Threonine
Number of rats	7	8	4	14	11	8
Body weight (kg)	0.227	0.223	0.213	0.236	0.235	0.247
Metabolic body size ($kg^{¾}$)	0.329	0.325	0.313	0.338	0.336	0.350
			Nitrogen balance data (mg N/day/$kg^{¾}$)			
"N-free" period						
N intake	5	4	4	4	4	4
Fecal N	42	38	41	39	38	30
Urinary N	140	125	138	156	147	115
Nitrogen balance	−177	−159	−175	−191	−180	−141
"Half-N" period						
N intake	75	75	80	76	79	76
Fecal N	37	37	39	36	36	28
Urinary N	109	142	138	122	170	147
Nitrogen balance	− 71	−104	− 97	− 82	−127	−100
"Full-N" period						
N intake	141	146	154	146	155	147
Fecal N	38	40	41	36	34	29
Urinary N	110	152	139	118	206	191
Nitrogen balance	− 5	− 46	− 25	− 10	− 85	− 73
K′ (Half-N; full-N)[1]	1.01 ± 0.10	0.82 ± 0.08	0.98 ± 0.05	1.04 ± 0.07	0.61 ± 0.09	0.38 ± 0.05
NI_e[2]	149 ± 11	213 ± 19	181 ± 4	157 ± 6	342 ± 42	380 ± 47

[1] K′ is the slope of the line joining the half-N and full-N points when nitrogen balance is plotted against nitrogen intake.
[2] NI_e is the nitrogen intake computed to be necessary for attainment of nitrogen equilibrium.

plies complete utilization of the increment of nitrogen obtained by doubling the nitrogen intake. If nitrogen balance is plotted against nitrogen intake, the slope of the line joining the two points representing half-N and full-N is equal to the fraction utilized of the increment in nitrogen intake. This coefficient of nitrogen utilization is the K′ of Allison et al. ('46). As can be seen in table 2, the values of K′ in both experiments with the complete amino acid mixture did not differ significantly from unity. Extrapolation of the line represented by K′ yields an estimate of the nitrogen required for equilibrium and this item is entered as NI_e in table 2. For the complete amino acid mixture both series of rats yielded essentially identical values for NI_e. Furthermore, the nitrogen balances in these two experiments agreed very well. According to these three criteria, the responses of the two series of rats to the ingestion of the complete amino acid mixture were identical.

Feeding the one-third DL-threonine mixture led to increased urinary nitrogen as compared with that found in the N-free period. K′ was reduced but the difference was not significant. NI_e was significantly increased ($P = 0.015$). The nitrogen balance was less favorable than with the complete mixture.

The mixture containing one-sixth L-threonine appeared to be slightly superior to that containing one-third DL-threonine on every count. K′ was not different from unity and NI_e was on the borderline of being different from NI_e for the complete mixture ($P = 0.065$).

The results from series 160 left some doubt as to whether the threonine in experiments II and III was reduced sufficiently to limit the utilization of the whole mixture. In series 240, therefore, threonine was reduced further, as is indicated in table 2. The feeding of mixtures containing either one-eighth L- or one-fourth DL-threonine yielded K′ values that were significantly smaller ($P < 0.003$) than the one obtained with the complete amino acid mixture. NI_e was more than doubled and nitrogen balances were decidedly negative. These facts leave no doubt that the threonine con-

centration, in these experiments, was the limiting factor in the utilization of the total nitrogen of the mixture. In series 240, as in series 160, the L-threonine mixture appeared to be slightly superior to the DL-threonine mixture, which contained the same amount of the L isomer. These results indicate that the D form is not utilized and may possibly interfere slightly with the attainment of nitrogen balance.

DISCUSSION

If the data from the low threonine experiments are considered in a slightly different manner, they all fall into a reasonably consistent pattern. In figure 1 nitrogen balance is plotted against threonine nitrogen intake. The average results from feeding L-threonine are represented by the solid symbols. The line of regression was computed from 30 individual experiments. Extrapolation of this line upward indicates that the requirement for the maintenance of nitrogen balance under these conditions is 2.36 mg of L-threonine nitrogen/day/$kg^{3/4}$. This is equivalent to 20.1 mg of the amino acid. Another method of arriving at the L-threonine requirement is to extrapolate the line joining each pair of points (half-N and full-N) to zero nitrogen balance. The mean requirement arrived at in this manner is 2.34 ± 0.09 mg L-threonine nitrogen/day/$kg^{3/4}$.

The average results from feeding DL-threonine are represented in figure 1 by the open symbols. Extrapolation of the line of regression for 32 experiments indicates a requirement of 5.34 mg of DL-threonine nitrogen/day/$kg^{3/4}$ for the maintenance of nitrogen equilibrium. The alternative procedure of extrapolating each pair of points yields a value of 6.16 ± 0.54. It is evident that D-threonine is not utilized by the adult rat under these conditions.

In figure 1 the L components of the DL-mixtures are represented by the half shaded symbols. They fall into line very satisfactorily with the points obtained directly by feeding the natural isomer. This lends strong support to the ac-

curacy of the estimated requirement of L-threonine. It is evident also that, under these conditions, nitrogen balance is a linear function of L-threonine intake.

Rose ('49) reported 0.50 gm of L-threonine/day as the requirement for the adult human male. If the average body weight of his subjects is assumed to be 70 kg, the requirement is 20.7 mg of L-threonine/day/kg$^{3/4}$. Thus the requirement of the adult male rat (20.1 mg/day/kg$^{3/4}$) as determined in the present investigation is the same as for man. In a previous

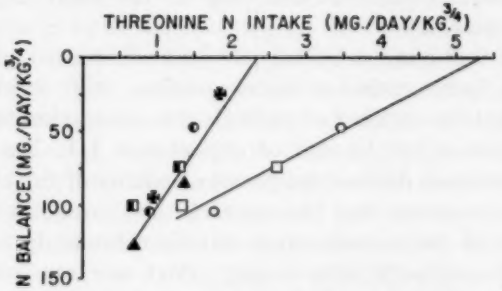

Fig. 1 Solid symbols refer to experiments with L-threonine and open symbols refer to experiments with DL-threonine. The half shaded symbols represent the L component only of the corresponding DL mixtures. ✠ = 1/6 L-threonine, half-N and full-N, series 160; ▲ = 1/8 L-threonine, half-N and full-N, series 240; 0 = 1/3 DL-threonine, half-N and full-N, series 160; □ = 1/4 DL-threonine, half-N and full-N, series 240. Nitrogen balance is negative in all experiments. See text for further explanation.

paper (Nasset and Anderson, '51) it was established that the requirement of methionine for the rat on the same basis is only 11% less than for man.

Benditt et al. ('50) state that the adult male albino rat requires 5.1 mg of threonine/day/100 cm^2 of body surface to maintain nitrogen equilibrium. Assuming that their average rat weighed 318 gm (mean of the limits given, i.e. 283 to 353 gm), the surface area, according to Lee's formula ('29), which they used, is 397.5 cm^2. According to their estimate, therefore, the daily requirement for this average rat is 20.17 mg of threonine. A body weight of 318 gm corresponds

to 0.423 $kg^{¾}$ and, using this unit of metabolic body size, the requirement is 47.7 mg of threonine/day/$kg^{¾}$, which is 137% greater than the value established in our investigation. Because of the small number of animals used, the validity of the requirement as published by Benditt et al. is open to serious doubt. They compounded a series of diets differing only in their content of threonine, and each of these diets was fed to a single animal. Only the few animals that remained close to nitrogen equilibrium contributed data for the graphic method used in arriving at the daily requirement for threonine.

Partial threonine deficiency, as induced in our experiments, produced some rather unusual results. It is evident from table 2 that the number of animals was approximately halved in each series by the end of experiment III. Many of the missing animals died as the result of edema of the esophagus. This was so severe that the stomach tube could not be passed and some of the animals were unable voluntarily to swallow any food and very little water. Post mortem examination showed that the lumen of the esophagus was usually narrowed in the middle portion to less than one-third of its normal diameter and often contained a plug of inspissated mucus. Even among the animals which survived all of the experiments, there were several in which it was very difficult to pass the stomach tube. It is rather surprising that this condition was noted as early as 4 days after beginning to feed diets low in threonine. The ascites and generalized edema found in the growing mouse on threonine-deficient diets (Bauer and Berg, '43) were not evident in our animals. Incipient edema may very well have been present in many tissues but it was probably induced in acute form in the esophagus by the passage twice daily of the stomach tube.

SUMMARY

Nitrogen balance was determined on two groups of adult male albino rats which derived all of their dietary nitrogen from mixtures of amino acids. Each experiment included the

following dietary regimes in the order given: 14 days on maintenance diet (9.6% whole egg protein); 7 days on N-free diet; 7 days on amino acid diet supplying approximately half of the maintenance requirement of total nitrogen; 7 days on double the quantity of the amino acid mixture fed in the previous period. These diets, except the maintenance diet, were fed by stomach tube in two equal portions daily, and each rat received the same quantity of diet each day.

At and below the limiting concentration, threonine intake is linearly related to nitrogen balance. The natural isomer of threonine is twice as effective as the racemic mixture and hence it is concluded that D-threonine cannot be utilized by the adult rat. The requirement of the rat, under these conditions, is computed to be 2.36 mg of L-threonine nitrogen/day/$kg^{3/4}$ which is equivalent to 20.1 mg of the amino acid. The requirement for man, recomputed on the same basis from the data of Rose ('49), is 20.7 mg of L-threonine/day/$kg^{3/4}$.

The rats on diets low in threonine developed edema of the esophagus to such an extent that it was difficult or impossible to pass the stomach tube. This condition developed rapidly and was the cause of death of several animals.

ACKNOWLEDGMENTS

Dr. Lee Crump of the Departments of Radiation Biology and Mathematics gave valuable advice on the mathematical treatment of the data. Mr. S. A. Pozzanghera, Miss June Palladino, Miss Margaret Ely, Miss Selma Hayman, Mr. Eugene Notz and Mrs. Andrea McCarthy gave skillful technical assistance.

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YELLOW FAT IN THE MINK[1]

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(Received for publication May 11, 1951)

A disease which mink ranchers have termed "yellow fat" has been encountered in many mink herds in the United States. This disease usually affects young mink rather than adult animals and occurs during the months of July, August, September and October, when the mink kits are from three to 6 months old and are growing rapidly. Often it is the largest and healthiest-appearing animals that develop the condition. A typical symptom of the disease is a partial paralysis of the hind quarters, first noticed when the mink acquire an unsteady gait. Finally the mink flatten out on the bottom of the cage and push themselves along on their abdomens. Mortality in the mink which show these symptoms is from 50 to 75%. Upon autopsy, a marked subcutaneous edema and a brownish yellow coloration of the depot fat are apparent.

Studies by Beadle, Wilder and Kraybill ('48) showed that yellow fat in hogs may be the result of feeding flaxseed, which contains 16% linolenic acid as measured by the spectrophotometric method. These workers also found that when sufficient flaxseed was fed to supply 14.5% linolenic acid in the diet of rats, up to 27.6% of the fatty acids present in the depot fat was trienoic acid. Recent data of Brooker and Shorland ('50) and Gupta and Hilditch ('51) show that horse fat contains as much as 16% linolenic acid. The data presented

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. These studies were supported by Project 614 of the Wisconsin Agricultural Experiment Station and grants from the Borden Co., New York City, and Fur Farm Food and Supplies, St. Paul, Minn.

in the present paper indicate that the "yellow fat" disease in mink may result from feeding horse fat or other feeds that contain high amounts of unsaturated fatty acids such as linolenic acid. The protective action of α-tocopherol is also shown.

EXPERIMENTAL AND RESULTS

A herd of 150 mink were fed a diet of the following composition: horsemeat 19%, liver 3.5%, a commercial cereal mix 12.5% and water 65%. This herd supplied the experimental animals and those used for control purposes. The experimental animals were chosen at random from the herd and given either raw linseed oil or raw linseed oil plus α-tocopherol in addition to the basal diet. In a preliminary experiment 6 mink kits were given the basal diet plus 5% and 6 mink the same diet plus 10% linolenic acid as raw linseed oil. Within 6 to 9 days all the kits exhibited symptoms of yellow fat disease, such as paralysis and a marked subcutaneous edema; however, the depot fat did not show any yellow coloration.

Since these high levels of raw linseed oil did not allow development of the yellow coloration of the depot fat before death, 10 young mink were fed 3% linolenic acid as raw linseed oil. At the same time the possible protective action of vitamin E was tested. The 10 mink were divided into 5 groups of two mink each. One group did not receive α-tocopherol, while the remaining 4 groups received daily supplements of 0.04, 0.57, 2.6 and 4.9 mg α-tocopherol, respectively. Vitamin E assay (Swick and Baumann, '50) showed that the basal diet already supplied 2.3 mg of vitamin E per mink per day, based on food consumption records.

One mink from the group receiving 0.57 mg and one from the group receiving no supplement of α-tocopherol died early in the experiment (13th day). These mink became paralyzed before death occurred. Upon autopsy a marked subcutaneous edema was apparent but no yellow coloration of the depot fat had occurred. Later (14th and 18th days) one mink receiving 0.4 mg and one receiving no supplement of α-tocopherol be-

came paralyzed and died. Autopsy revealed marked subcutaneous edema and yellow coloration of the depot fat. The first mink to show this condition had localized spots of yellow fat in the abdominal depot fat, while the other mink exhibited a deep yellow coloration throughout the depot fat of the body. Five mink, one receiving 0.57 mg, two receiving 2.6 mg, and two receiving 4.9 mg α-tocopherol, appeared normal throughout the 12-week experiment and when killed did not show symptoms of yellow fat disease. One mink receiving 0.44 mg α-tocopherol refused to eat early in the experiment and died.

In another experiment the feeding of linolenic acid as raw linseed oil at an 8% level for 5 months did not produce yellow fat symptoms in 6 adult mink.

The amount of trienoic acid deposited by mink is affected by dietary fats (table 1). High amounts of trienoic acid were deposited when the basal diet containing 19% horse meat (10% fat) and raw linseed oil was fed. High amounts of trienoic acid also resulted when a ranch diet containing 19% horse meat (17% fat) was given. In contrast, low amounts of trienoic acid were deposited when the basal diet containing 19% horse meat (10% fat) was fed without linseed oil. Very low amounts of trienoic acid are deposited when a purified diet (Tove, Lalor and Elvehjem, '50) containing 11% cottonseed oil as the source of dietary fat, is fed. These analyses were made by the spectrophotometric method (Spectroscopy Committee, American Oil Chemists Society, '49).

The range of values obtained for the amounts of dienoic, trienoic and tetraenoic acids in 6 different samples of horse fat is also given in table 1.

DISCUSSION

It is evident that yellow fat disease can be produced in young mink by feeding raw linseed oil with a horse meat-cereal (basal) diet. This may mean that horse fat, which contains high amounts of linolenic acid, is the cause of yellow fat disease on mink ranches. It should be noted that mink acquired yellow fat on a ranch while receiving a diet identical to the basal diet

used in this experiment, except that the ranch diet contained horse meat which contained 7% more fat than the horse meat in the basal diet. Yellow fat disease is encountered frequently when diets containing fish are fed to mink. This may be explained by the fact that fish contain high amounts of unsaturated fatty acids.

TABLE 1

Per cent C_{18} unsaturated fatty acids in mink and horse fat

SAMPLE NO.	ACIDS		
	Dienoic	Trienoic	Tetraenoic
Fat from mink exhibiting yellow fat (basal diet plus raw linseed oil)			
1	9.5	9.4	1.2
2	9.6	11.3	1.1
Fat from mink exhibiting yellow fat (ranch diet, same as basal except 7% more horse fat)			
1	10.0	6.9	1.3
2	6.8	5.0	1.1
Fat from normal mink (basal diet)			
1	10.3	4.7	0.55
2	14.4	2.8	0.91
Fat from normal mink (purified diet containing cottonseed oil)			
1	24.8	0.85	0.55
2	28.4	1.1	1.1
Horse depot fat (ranges of 6 different samples)	3.5 to 12.6	9.4 to 17.0	0.42 to 0.86

The protective action of α-tocopherol is demonstrated in these studies. Whether the protection is afforded by alleviation of a vitamin E deficiency brought on by unsaturated acids, or whether the α-tocopherol is acting as a detoxifying agent, is not known. Often horse meat that has been stored for a considerable time is being fed to mink when yellow fat disease occurs. This could be the result of the destruction of vitamin E during storage.

A yellow pigment has been encountered by workers (Filer, Rumery and Mason, '46) dealing with vitamin E deficiency

in the rat. These workers suggest that the pigment may be polymerized peroxides of unsaturated fatty acids. If this is correct, perhaps peroxides formed in horse fat during storage accentuate the ability of the fat to produce yellow fat disease in mink.

The type and amount of dietary fat determine the amount of trienoic acid appearing in the depot fat of the mink. The amount of trienoic acid deposited parallels the amount of the acid fed.

SUMMARY

1. Yellow fat in the mink may be the result of feeding relatively high amounts of trienoic acid, which is present in horse fat to the extent of 16%.

2. The feeding of 3% linolenic acid as raw linseed oil produced yellow fat in mink.

3. α-Tocopherol seems to exert a protective action under our experimental conditions.

ACKNOWLEDGMENTS

We are indebted to Dr. R. M. Shackelford, Department of Genetics, University of Wisconsin, for his cooperation and general interest in these studies; and to Dr. A. M. McDermid, Middleton, Wisconsin, and Dr. M. Savan, Department of Veterinary Science, University of Wisconsin, for the autopsies performed in connection with these studies.

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CALORIES — A LIMITING FACTOR IN THE GROWTH OF CHILDREN

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THREE FIGURES

(Received for publication April 23, 1951)

It is generally assumed that an adequate food intake is synonymous with satisfactory nutriture and desirable growth in children, although few concurrent measurements of food consumption and growth have been reported for healthy active children. Data resulting from accumulated food records, which may or may not represent the customary, freely-chosen diets of children, and evaluations based on current incomplete tables of food composition, have constituted the basis for estimating dietary allowances for many years, although they are recognized to possess inherent inaccuracies and many shortcomings. Likewise there are many studies and reports of cross-sectional and longitudinal data on anthropometric measurements in childhood, which possess no accompanying information concerning the quality or quantity of food which influenced the growth status observed.

Physical growth may be defined as the series of anatomic and physiologic changes taking place with increasing age from prenatal life through infancy, childhood, and adulthood. It may be considered in terms of both visible and invisible growth. Increase of body dimensions or their anatomic

changes, and body weight, are visible and therefore are subject to direct measurement. Invisible growth, on the other hand, may occur with progressive maturation and may be measured indirectly by the absorption and storage in the body of structural and functional constituents obtained from food eaten, by basal metabolic rate determinations and other physiologic measurements and by roentgenographic recordings. A detailed study of invisible growth, though necessarily accomplished by indirect procedures, contributes information pertinent to a better understanding of the quantity and quality of the chemical increments necessary for growth in both of its aspects.

The results of a comprehensive investigation [1] of both the visible and invisible growth of a group of children during a period of some 15 years have recently been published from this laboratory (Macy, '42, '46, '51a). The critical food and feeding problems consequent to our national efforts toward world peace and the alleviation of malnutrition have impelled assembly of this preliminary report on the dynamics of energy and protein metabolism in growth, using data which permit comparison of the energy intakes of some of the subjects

[1] The organization of the investigation, selection of average healthy children, procedures employed in making observations and measurements, and methods used in analyses of food, urine, feces and blood were given in detail in the first volume of the report on nutrition and chemical growth in childhood. The original data obtained from the thousands of determinations of many kinds were presented in the second volume, and the results of such calculations as might provide useful information were given in a third. Space limitations preclude repetition of details of procedure, but it is essential to remember that the children studied were judged representative of the normal by numerous standards, and that every effort was expended to conduct the studies with minimum interference with healthy living in congenial surroundings. The energy intakes were determined as heat of combustion in a calorimeter and physiologic fuel values were calculated by subtracting the values obtained by determining the heat of combustion of the feces and urine excreted. Basal metabolism measurements were obtained in the morning before arising and the children were weighed every 5th morning on a Troemer beam balance accurate to 0.2 gm. Nitrogen retentions were calculated from results of chemical determinations of the amounts in the food intake, in the feces and in the urine.

with their basal metabolic requirements, storage of nitrogen, and weight gains during consecutive periods of time.

EXPERIMENTAL

Establishing adequate food intakes for children

For three months preliminary to a study of 10 healthy children 4 to 9 years old for 45 consecutive 5-day balance periods, every effort was made to assure each child an adequate diet, including one quart of milk daily. For at least two weeks immediately preceding the investigation each child received a diet carefully planned to meet all known chemical requirements. The diets of all children were composed of the same common foods, and during the pre-investigation interval levels of food intake were adjusted to fulfill the demands of hunger. Daily fluctuations in appetite were provided for by freedom in intake of pure carbohydrate, provided all other food served was eaten. The plan was to continue the pattern dietary for each child during succeeding months, but analytical results soon demonstrated that for some subjects the voluntary intakes of food were not providing sufficient calories for satisfactory weight gains. This chance situation in connection with continuous measurements, concurrently, of visible and invisible growth permits some insight into the influence of level of calorie intake upon the growth of children, even when they are consuming diets adequate with respect to other known dietary essentials.

The periods of inadequate intake of calories did not exist for sufficient time to produce a significant loss of body tissue, and increased food intakes were followed immediately by rapid weight gains. In general, protein intakes were increased by provision of increased energy intakes and the percentages of total calories provided by protein were fairly constant (14 to 16% of the physiologic fuel value). The biological evaluation of dietary adequacy by relating energy intake to weight increase and to nitrogen storage emphasizes that neither appetite nor any of the known standards can be relied upon to provide adequate food intakes for individual children.

Nitrogen storage and growth

If the potentialities of the growth impulse are to be realized in full measure, the diet must provide adequate calories, proteins with a satisfactory mixture of amino acids for fabrication of protoplasmic tissue, and minerals and vitamins for construction of body tissue and maintenance of body function. The results of determinations of energy and nitrogen utilization and body weight gain reflect the influence of factors that modify the changes in structure and function concurrently

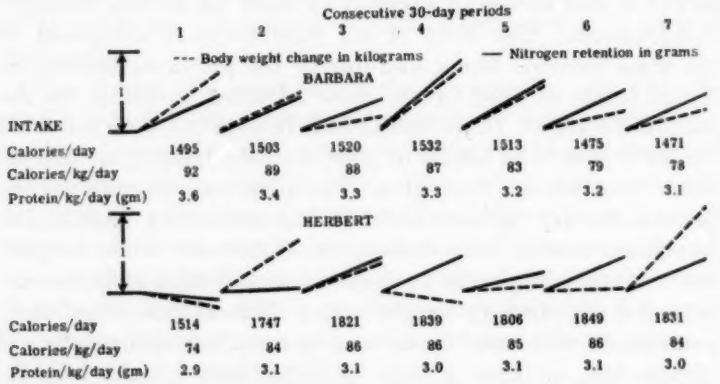
Consecutive 30-day periods
1 2 3 4 5 6 7
--- Body weight change in kilograms
— Nitrogen retention in grams
BARBARA
INTAKE
Calories/day 1495 1503 1520 1532 1513 1475 1471
Calories/kg/day 92 89 88 87 83 79 78
Protein/kg/day (gm) 3.6 3.4 3.3 3.3 3.2 3.2 3.1
HERBERT
Calories/day 1514 1747 1821 1839 1806 1849 1831
Calories/kg/day 74 84 86 86 85 86 84
Protein/kg/day (gm) 2.9 3.1 3.1 3.0 3.1 3.1 3.0

Fig. 1 Weight changes and average daily nitrogen retentions of two children during each of 7 consecutive 30-day periods.

taking place in the growing child's body. Such data are illustrated for two children in figure 1.

Biochemical individualities may be modified by both the rate and quality of growth, and thereby influence the metabolic needs at a given time. If the growth impulse is a constant force, the "change in size" which constitutes visible growth seldom manifests constancy, and the vicissitudes of existence are known to present variable requirements for repair which are superimposed upon those for maintenance and growth. Under usual circumstances the human body will accumulate, from a dietary ample in all respects, reserves of some nutri-

ments to provide for periods of stress which create increased demands. Too, growth may be limited by "conditioning" resulting from the body's adaptation to an insufficient or improper diet over a considerable period of time, and increases in body mass may consist largely of water and adipose tissue instead of protoplasmic tissue.

Among the children studied, retention of nitrogen for tissue construction was depressed when the calorie intake level was insufficient to promote satisfactory gain in body weight, especially in the initial observation period. Sometimes rate of increase in body weight exceeded by far the accretion of nitrogen; at other times there was no gain in body weight for a time although nitrogen was being retained. The data in figure 1 illustrate these changes for two children. Before and throughout 7 consecutive months of study Barbara showed steady weight gains and retentions of nitrogen, although her intakes of calories and protein per kilogram of body weight consistently declined. Herbert, however, lost both weight and nitrogen during the initial periods of study but an increase in calorie intake by 10 Cal. per kilogram of body weight was followed by nitrogen retention and weight gain. Later in the study, without appreciable change in intake of calories or protein, Herbert showed a loss of body weight without a decline in nitrogen retention.

Although changes in body mass may not be a true measure of chemical growth, it seems significant that in healthy, well-nourished children as few as 10 Cal. per kilogram of body weight spelled success or failure in making satisfactory weight gains, when intakes of protein per kilogram of body weight approximated 3 gm and protein furnished from 14 to 16% of calorie intakes. Ill and less fortunate children, with poorly nourished bodies, vary more widely in their calorie and protein needs, and for them a small deficit in calorie intake may have far-reaching detrimental effects, not only with respect to body weight but also upon tissue formation and physiologic function. The circumstances which promoted growth in these children as well as those which inhibited it

temporarily are being explored more fully in relation to mineral metabolism and skeletal maturation (Macy, '51b).

Determination of adequate calorie intake

The criterion of adequate energy intake for a child at any time, and for special groups of children, must be based upon growth rather than upon compliance with dietary allowances for population groups or standards developed from dietary consumption records without sufficient knowledge of nutrition and progress of growth. The importance of interpreting individual caloric needs in terms of their own growth rate was summarized meaningfully by the Committee on Growth and Development of the White House Conference on Child Health and Protection ('32):

"It should be remembered that each individual child has his own energy needs which may vary from that of others of the same age and size. This optimum is such that the child grows properly, feels well, is mentally and physically alert and adjusts himself to his environment. Standards of food intake are of value in improving the welfare of children, but should not be used to exclusion of the consideration of the problem of the individual."

Similar energy intakes by different children of like age and weight do not result in like gains in weight, nor do like energy intakes in the same children at different times promote the same increases in weight. With living beings observations cannot be duplicated under the same endogenous and exogenous conditions, and the apparent calorie requirement of a child may vary markedly with different intensities of the growth impulse. The available energy which the healthy child consumes in excess of his basal and activity requirements determines, in large measure, the rate of increase in body weight: an insufficiency may limit growth, a surplus may produce obesity.

Energy intakes of 50 to 100% above the basal metabolism level, depending on activity, are alleged to meet the requirements of metabolism and activity for healthy growing children

under usual circumstances. Maroney and Johnston ('37), studying relatively inactive, hospitalized children, found that the increment for energy above the basal requirement approximated 74% for boys and 67% for girls. For the 10 children considered in the present paper twice the basal metabolism per 24 hours in most instances exceeded the determined physiologic fuel value of the food intake, and for the majority of the children exceeded the heat of combustion of the intake. Illustrative values for two children are shown with those for

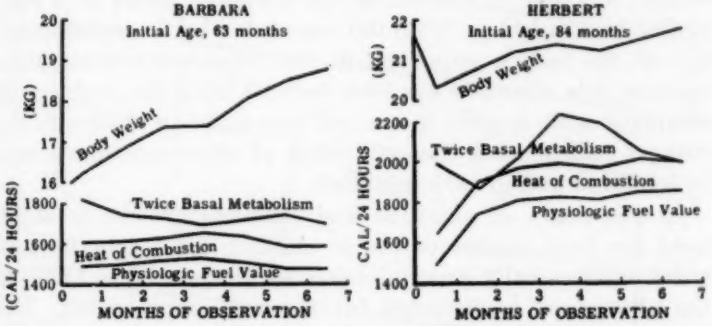

Fig. 2 Cumulated weight gains and average daily energy intakes of two children during each of 7 consecutive months. Initial body weight is indicated at juncture of weight curves with vertical axis.

body weight during the 7 months of the study in figure 2. One child (Barbara) made steady and adequate weight gains throughout the study with constantly decreasing energy intake per kilogram of body weight; the other child (Herbert) lost weight initially and during the 5th month of the study. It seems evident that an allowance of foods providing calories in quantities double the basal metabolism per 24 hours would be ample for most children, whether the calorie intake was determined or calculated on the basis of either heat of combustion or physiologic fuel value.

Recommended allowances

The allowances recommended by the Food and Nutrition Board of the National Research Council ('48) have been widely

used in this country as dietary standards, although in presenting them the Board stressed that the amounts recommended are not "requirements" and "the allowances are intended to serve as a guide for planning an adequate diet for every normal person of the population and not merely for the average member of the group categories." Others (Beal, Burke, and Stuart, '45; Keys, '49–'50; Darby, '47; Pett, '51) have also reiterated that many factors — genetic, physiologic, and environmental — must be considered with age, weight, and size in evaluating the nutritive needs of a particular human being. With the search for better understanding of the body's requirements for other nutriments than calories, less attention has been focused upon the problem of adequacy with respect to energy, especially in children; yet without sufficient calories, utilization of other substances may be impaired and growth inhibited.

In this study of nutrition and chemical growth in childhood the food intakes of the 10 children for 7 months provided average daily energy intakes which were 95 to 113% of the allowances recomended for the ages represented. Per kilogram of body weight, the daily energy intakes over the entire 7 months ranged from 89 to 108% of the recommended amounts. In figure 3 the mean energy intakes of each boy per kilogram of body weight during each of the 7 consecutive 30-day periods are plotted against the respective changes in body weight during the periods. The "physique channel" of each child according to evaluation with the Wetzel Grid (Wetzel, '41) is given in parenthesis following the name. Superimposed on the graph are lines indicating the allowances of calories recommended per kilogram of body weight by the Food and Nutrition Board, assuming a straight-line relationship.

The curves in figure 3 demonstrate the unsatisfactory weight gains of some of the children during the early weeks of the study, followed by improvement with increased intake of calories per kilogram of body weight. The children with the least satisfactory weight gains and large increases in calorie

intake voluntarily maintained intakes higher than at the start of the study, although all of the children decreased their levels of intake during the latter months of the study, while continuing to gain weight. Differences in type and speed of growth are also, of course, reflected in the curves. Increases of 4 to 11 Cal. per kilogram of body weight in the average daily

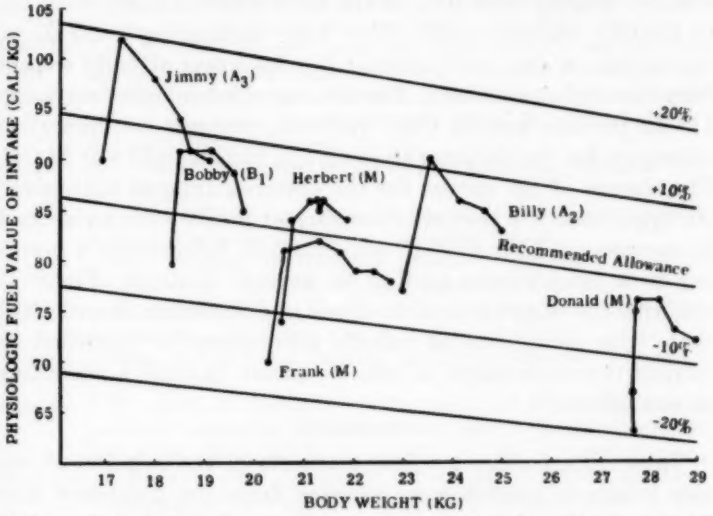

Fig. 3 Mean daily energy intakes per kilogram of body weight versus body weight for 6 boys during consecutive months. Wetzel Grid channels are indicated in parentheses. The recommended allowances for children and variations of ± 10 and 20% are also shown per kilogram of body weight. For 4 girls in the study group, corresponding curves were almost wholly within ± 10% of the recommended allowances, with placement on the Wetzel Grid either in channel M or A_3.

intake of calories during the second month of the study were accompanied by weight gains for the children who had showed slight losses of weight during the preceding 30 days, demonstrating that a difference in intake of only a few calories per kilogram of body weight per day may mean nutritive success or failure, even when the dietary is excellent in providing essential nutriments.

The validity of the recommended allowances as a guide in planning adequate intakes of calories for *groups* is supported by the distribution of the curves in figure 3 in relation to the Food and Nutrition Board recomendations. Objections to the levels advocated as being unnecessarily high are not substantiated with respect to energy intake by these results. During over 40% of the 69 months of study with the 10 healthy children while they were consuming good diets, the calorie intakes were greater per kilogram of body weight than the recomendations. The average physiologic fuel value for all periods was 82 Cal., while the average recommended allowance for the children according to body weight was 84 Cal. The shapes of the curves for the children suggest that, given the opportunity, a period of insufficient intake of calories may be compensated for by high consumption, followed by a lowering to a level corresponding to normal demand. Figure 3 confirms the suggestion of the Food and Nutrition Board ('48) that "the recommended calorie allowances be regarded as subject to modifications of plus or minus 15 to 20% according to conditions."

SUMMARY

Values illustrating pertinent aspects of the relation of calorie intake to growth were selected from the published data of a comprehensive study of nutrition and chemical growth in childhood. The values demonstrate:

1. Wide variation in the growth progress of the same child at different times and of different children at the same time.
2. That appetite may not be relied upon to assure ingestion of quantities of a good mixed diet which will provide sufficient calories for satisfactory weight gains.
3. That existing "standards" may not be depended upon in every instance as criteria of dietary adequacy.
4. A depression of nitrogen retention when intake of calories is insufficient to permit proper gain in body weight.
5. That invisible growth may occur, as evidenced by nitrogen retention, coincident with failure to accomplish satisfactory gains — or even with a loss — of body weight.

6. That a difference in intake of as few as 10 Cal. per kilogram of body weight may spell success or failure in making satisfactory progress in visible and invisible growth.

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THE CALCIUM BALANCE OF ADULT HUMAN SUBJECTS ON HIGH- AND LOW-FAT (BUTTER) DIETS[1]

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ONE FIGURE

(Received for publication April 28, 1951)

Conceivably, dietary fat may affect calcium metabolism and calcium utilization in either a favorable or an unfavorable fashion. Dietary fat on hydrolysis in the alimentary tract may form soaps with calcium. These soaps may be so insoluble as to increase the fecal calcium and augment calcium wastage in digestion, or they may enter into complexes with bile salts, the solubility and absorbability of which by the intestinal mucosa are greater than those of the calcium soaps themselves (Adler, '27; Oelsner and Klinke, '29; Beznák, '31). Whether the effect goes in the one direction or in the other will depend upon the nature of the fatty acids involved, and specifically upon their digestibility (Boyd, Crum and Lyman, '32) which is a function of their melting points (Steenbock, Irwin and Weber, '36; Hoagland and Snider, '43; Crockett and Deuel, '47). Adler ('27) has shown that bile increases the solubility of the mixed fecal calcium soaps from human subjects with obstructive jaundice. The inability of Langley, Rosenbaum and Rosenbaum ('32) to demonstrate an appreci-

[1] The investigations reported in this paper were aided by funds contributed to the University of Illinois by the Nutrition Foundation, Inc., of New York City.

able solubility of calcium stearate in bile may have been due to the chemical properties of stearic acid itself, or, as the authors suggest, to the selection of an unfavorable bicarbonate concentration of the solution mixture, an item of considerable importance according to Adler ('27). The close interrelationships existing between calcium salts and fats in the digestive tracts of animals have been well demonstrated in the early work of Givens ('17) on dogs and in the recent report of Cheng, Morehouse and Deuel ('49) of experiments on rats, not to mention the many reports on the antirachitic effects of dietary fats, which have been reviewed by Jones ('40).

Intestinal acidity seems to favor the absorption of calcium salts, according to Irving and Ferguson ('25) in a report on experiments on dogs and rabbits, and in the experience of Jones ('42) lard and oleic acid increased the acidity of the cecum and colon of rats. However, Boyd, Crum and Lyman ('32) have observed that whether fats increase the acidity of the intestinal tract or not depends upon the nature of the calcium salts with which they are fed: the addition of fat to a diet containing calcium chloride definitely lowered the pH of the intestine and the feces, but when fat was added to a diet containing calcium lactate, the pH was elevated slightly.

French ('42, '43) has reported carefully planned feeding experiments with rats designed to study the interrelations of calcium and fat utilization. Oleo oil (m.p. 41.5°C.) was fed at different levels to rats receiving equalized caloric intakes. There was a moderate decrease in calcium utilization with increasing proportions of fat in the diet, associated with a moderate increase in the pH of the intestinal contents. When the proportion of fat was increased to 45% of the diet, the impairment in calcium utilization in one test was severe.

The only experiment that the present authors were able to find on adult human subjects, in which a specific study was made of calcium retention on high- and low-fat diets, is that

of Mallon, Jordan and Johnson ('30). Two healthy young women served as subjects in a metabolism period of 18 days' duration. The conclusion of the authors is well founded on the experimental data secured: "As the subjects shifted from a positive to a negative calcium balance or *vice versa* within two 3-day periods it would seem that fat *per se* cannot be said to have exercised a definite influence upon the calcium retention of the two subjects of this study."

TABLE 1

Description of experimental subjects

SUBJECT	NO. OF TESTS	AVERAGE AGE	AVERAGE BODY WEIGHT	HEIGHT
		yrs.	*kg*	*cm.*
F.R.S.	3	44	82.7	180
J.S.	2	24.5	74.5	181
J.H.	2	19.5	67.5	167
A.B.	1	20	67.5	167
C.R.	1	20	65.8	180
D.G.	1	20	76.2	...
E.M.	2	25	75.5	186
G.J.	2	29.5	78.0	176
H.V.	2	30	66.9	171
H.M.	1	27	63.9	175
R.S.	2	27.5	67.0	185
K.H.	2	29.5	66.5	175
R.H.	1	28	78.0	183

The experiments to be reported in this paper were designed to study the effect of a particular fat, milk fat, on the calcium metabolism of 13 adult human subjects. The experiments extended over a period of 4 years (from the spring of 1945 through the fall of 1949) and involved the testing of diets varying in fat content on the dry basis from 1 to 32%. Analysis of the results secured failed to reveal any effect of dietary fat, of the type used and within the range of intake employed, upon the calcium utilization of men. The description of the experimental subjects is given in table 1.

EXPERIMENTAL PROCEDURES

The plan of the experiments involved comparisons of the calcium balances of the subjects in alternating periods of high-fat and low-fat feeding. The experimental periods were generally 20 days long, though only the metabolism data of the last 16 days are presented and analyzed. For shorter periods than 20 days, the data of the first 4 days have been disregarded. The level of calcium intake varied in different periods from 2.71 to 10.10 mg per kilogram of body weight; two-thirds of the intakes were within the range of 4 to 7 mg per kilogram of body weight. The latter range of calcium intake was associated with negative calcium balances except in two out of 39 periods. Calcium intakes above 7 mg per kilogram were associated with positive calcium balances in 12 out of 15 periods.

For the first year and a half of the investigation, the basal diet consumed in all periods contained low-fat low-calcium foods: oatmeal, whole wheat bread, lean meat, oatmeal cookies, corn-starch pudding, potatoes, beets, prunes, apples and orange juice, with an adequate vitamin D supplement (1,000 I.U.). Calcium and adequate calories were provided by homogenized milk and butter for the high-fat diets, and by skim milk and sugar for the low-fat diets. For the remainder of the study, the basal diets contained skim milk; high-fat and low-fat modifications were made by appropriate combinations of butter and of sugar and honey. During the spring of 1947, commercial canned apple butter was used in the low-fat diets to provide in part the calories equivalent to the butter contained in the comparable high-fat diets. On analysis, the low-fat diets were found to contain appreciably more calcium than the high-fat diets, a discrepancy that was traced to the apple butter. This series of tests was not used to determine the effect of fat on calcium utilization, but the data were included in the determination of the regression of calcium balance on calcium intake.

During the springs of 1946 and of 1949, low-Ca basal diets, both high-fat and low-fat in character, were used in some periods for the more precise computation of calcium utilization values.

Composite samples of all diets were analyzed for calcium, moisture, ether extract and nitrogen. The feces and urine were analyzed for Ca. The details of the collection, sampling and analysis of diets, feces and urine are described in previous publications (Steggerda and Mitchell, '39, '46).

DISCUSSION OF THE EXPERIMENTAL RESULTS

The average data for the different subjects and experimental periods are assembled in table 2. The averages of 23 high-fat feeding periods expressed in milligrams of calcium per kilogram of body weight per day were: intake 6.71, and balance — 0.515. For 22 low-fat feeding periods, the average calcium intake on the same basis was 6.25, and the balance — 0.538. The difference in calcium balance between the two series of results is insignificant statistically, but the interpretation of the difference is complicated by the differences in calcium intake, averaging 0.46 mg of calcium per kilogram of body weight in favor of the high-fat feeding. It is also worth noting that the percentage of the total calcium excreted that appeared in the feces was not shown to be affected by the level of fat in the diet, the percentage averaging 69.7 on the low-fat regime and 71.7 on the high-fat regime.

On the assumption that the regression of calcium balance on calcium intake is unaffected by the proportion of fat in the diet, and in view of the fact that differences in calcium intake are largely due to differences in the amount of milk calcium consumed, the equation of a straight line was fitted by the method of least squares to all of the data secured, including the data obtained in the spring of 1947, not included in table 2. Figure 1 shows the scatter of the data around the regression line, the equation for which is

$$y = -3.72 + 0.502\ x,$$

TABLE 2

Calcium metabolism data

SUBJECT	CHARACTER OF DIET [1]	FAT CONTENT OF DIET ON DRY BASIS	LENGTH OF COLLECTION PERIOD [2]	DAILY CALCIUM PER KG BODY WT.		FECAL CA IN TOTAL EXCRETION
				Intake	Balance	
		%	*days*	*mg*	*mg*	%
1945 — Autumn						
F.R.S.	Low-fat	6	16	5.68	−0.32	84
	High-fat	26	12	6.34	−0.13	82
	Low-fat	6	8	5.68	+0.16	82
J.S.	High-fat	28	16	8.36	+0.68	79
	Low-fat	1	16	7.90	+0.99	73
	High-fat	28	12	8.04	−0.74	78
J.H.	High-fat	28	16	10.10	−0.16	59
	Low-fat	1	16	9.27	+2.41	66
	High-fat	28	12	9.58	+1.86	69
A.B.	High-fat	28	16	9.18	+0.06	64
	Low-fat	1	16	8.24	+1.46	69
	High-fat	28	12	8.62	+1.51	78
1946 — Spring						
J.S.	High-fat basal [3]	19	16	4.48	−1.37	70
	High-fat	23	16	6.56	−0.48	70
	Low-fat	1	16	5.57	−1.08	74
	Low-fat basal [3]	1	16	3.90	−2.70	77
	High-fat	23	16	6.41	−0.60	80
	Low-fat	1	16	7.47	+0.30	79
	Low-fat basal [3]	1	16	5.11	−1.28	78
C.R.	High-fat basal [3]	19	16	5.84	−0.80	73
	High-fat	23	16	8.22	−0.70	69
	Low-fat	1	16	6.49	−1.85	64
J.H.	High-fat basal [3]	19	16	5.65	−0.04	53
	High-fat	23	16	7.97	+0.72	60
	Low-fat	1	16	6.83	−0.72	57
	Low-fat basal [3]	1	16	4.96	−0.56	58
	High-fat	23	16	8.02	+1.74	63
	Low-fat	1	16	8.28	+1.99	66
	Low-fat basal [3]	1	16	5.65	+0.24	63
D.G.	Low-fat	1	16	7.24	+0.09	60
	Low-fat basal [3]	1	16	4.96	−0.41	63
1946 — Autumn						
E.M.	High-fat	25	16	5.11	−1.61	71
	Low-fat	11	12	5.70	−1.47	73
J.P.	High-fat	28	16	4.60	−1.27	68
	Low-fat	6	12	5.32	−1.07	69
G.J.	Low-fat	9	16	5.57	−1.20	81
	High-fat	27	12	5.21	−1.08	80

TABLE 2 *(continued)*

Calcium metabolism data

SUBJECT	CHARACTER OF DIET [1]	FAT CONTENT OF DIET ON DRY BASIS	LENGTH OF COLLECTION PERIOD [2]	DAILY CALCIUM PER KG BODY WT.		FECAL CA IN TOTAL EXCRETION
				Intake	Balance	
		%	*days*	*mg*	*mg*	%
1946 — Autumn *(continued)*						
H.V.	Low-fat	10	16	6.49	—1.48	72
	High-fat	26	12	6.76	—0.92	69
1947 — Autumn						
H.M.	Low-fat	7	16	5.34	—1.98	64
	High-fat	29	16	5.59	—2.72	71
R.S.	Low-fat	3	16	4.84	—1.21	62
	High-fat	32	16	5.07	—1.84	67
K.H.	High-fat	26	16	4.31	—2.28	72
	Low-fat	4	16	5.13	—2.22	73
1948 — Spring						
K.H.	High-fat	26	16	5.08	—1.72	79
	Low-fat	6	16	5.18	—2.39	72
R.S.	Low-fat	3	16	5.36	—0.49	69
	High-fat	30	8	4.83	—1.04	76
	High-fat basal [3]	27	16	2.86	—1.86	56
1949 — Spring						
F.R.S.	High-fat basal [3]	21	16	2.71	—1.48	67
	High-fat	21	16	4.82	—1.07	73
	High-protein, low-fat	14	16	4.56	—1.36	69
	High-fat	30	16	4.85	—0.69	82
R.H.	High-fat basal [3]	21	16	2.59	—1.76	38
	High-fat	21	16	4.99	—0.66	52
	Low-fat	4	16	5.42	—0.38	56
	High-fat	30	16	5.55	—0.44	62
Averages [4]	Low-fat			6.25	—0.538	69.7
	High-fat			6.71	—0.515	71.7

[1] The basal diet, through the spring of 1946, consisted of low-fat low-calcium foods: oatmeal, whole wheat bread, lean meat, oatmeal cookies, corn-starch pudding, potatoes, beets, prunes, apples, orange juice, vitamin D. Calcium and adequate calories were provided by homogenized milk and butter for the high-fat diets and skim milk and sugar for the low-fat diets. After the above date, the basal diet contained skim milk; high-fat and low-fat modifications were made by appropriate combinations of butter and of sugar and honey.

[2] Each collection period was preceded by a transition period of 4 days on the same diet.

[3] These diets contained no milk.

[4] Omitting high-fat and low-fat basals.

in which y is the calcium balance and x the calcium intake, both expressed in milligrams per day per kilogram of body weight.[2]

When the calcium balances of adjacent low-fat and high-fat feeding periods were corrected for differences in calcium intake, according to this equation, the average balances for 20 such comparisons were —0.567 and —0.728 mg of calcium

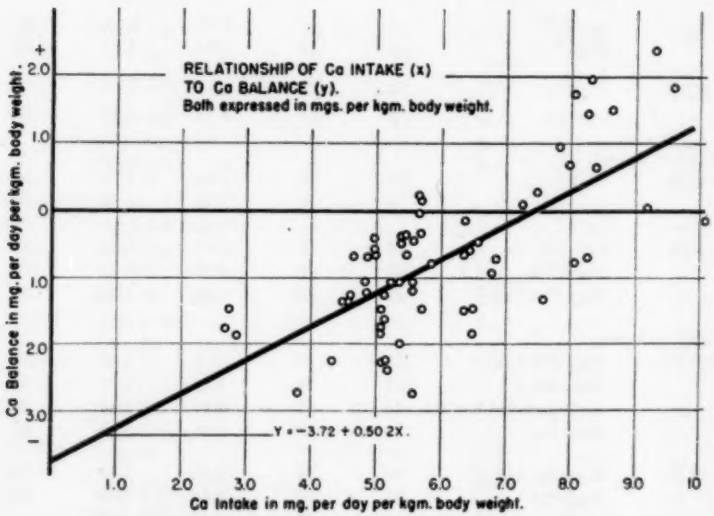

Fig. 1 The relationship between the calcium intake and the calcium balance of the subjects, both expressed in milligrams of calcium per kilogram of body weight per day. The data have been pooled regardless of the fat content of the diets.

[2] Longwell ('41) has presented evidence that among different species of animals, varying in size from the rat to man and to the pig, the endogenous calcium excretion varies with body surface or basal calories, rather than with body weight. One might expect, therefore, that calcium requirements for maintenance, in terms of dietary calcium of some particular description, would also be more closely related to body surface and to basal metabolism than to body weight. However, Steggerda and Mitchell ('46) have shown that, for a moderate range in body weight among adult men, requirements for calcium expressed per unit of body weight are no more variable than requirements expressed per unit of body surface.

per kilogram of body weight for the low-fat and high-fat tests, respectively. However, of the 20 individual comparisons, 10 favored the high-fat diet and 10 the low-fat diet. This comparison, also, fails to reveal an effect of fat on calcium utilization.

The constant, 3.72 in the above equation, is an estimate of the endogenous excretion of calcium in milligrams per kilogram of body weight per day, and may be compared with the value of 3.09 derived in a similar manner by Mitchell and Curzon ('39) from experimental results of a more heterogeneous character published in the literature.

The constant 0.502, measuring the slope of the regression line, indicates an average percentage utilization of dietary calcium in these experiments of 50.2, with a standard error of 6.2 (Rider, '39, p. 94). This is a higher average percentage utilization than that deduced by Mitchell and Curzon, 32%, and that observed by Steggerda and Mitchell with 19 adult men, also 32%. The difference of 18 percentage units seems to be greater than can be accounted for by errors of sampling. The value of 50.2%calcium utilization derived from the regression equation is in fair agreement with an average value of 41% derived from 8 tests (table 2) in which low-calcium basal diets were fed, permitting computations of calcium utilization. In this discussion the term "utilization" means the change in calcium balance in milligrams per 100 mg change in calcium intake.

If the value of y in the regression equation becomes 0, then the calcium intake, x, necessary for calcium equilibrium is 7.40 mg per kilogram body weight per day. This value is to be compared with the value of 9.21 reported by Steggerda and Mitchell ('46). The cause of the discrepancy is not obvious. It may in part be traceable to variation among subjects in their response to identical conditions, in part to variation within subjects on the same diet, and in part to differences in diets.

SUMMARY AND CONCLUSIONS

Calcium metabolism studies have been reported on 13 adult men, ranging in age from 19 to 44 years, involving 50 experimental periods of 12 to 20 days' duration. The main purpose of the investigation was to study the effect of variable dietary levels of milk fat, from 1 to 32% on the dry matter basis, on the utilization of dietary calcium, an average of 47% of which was derived from milk.

For the type of dietary fat studied and the range of intake employed, no effect on calcium metabolism was noted, either in the amount retained or in the proportion of excreted calcium found in the feces.

On pooling all of the data together, expressed in milligrams of calcium per day per kilogram of body weight, it was found that, under the conditions imposed, the regression of calcium balance (y) on calcium intake (x) is represented by the equation: $y = -3.72 + 0.502\ x$. This equation implies an average calcium utilization of $50.2 \pm 6.2\%$, a requirement of 7.40 mg of calcium per kilogram body weight per day for calcium equilibrium, and an endogenous output of calcium by adult men of 3.72 mg per kilogram of body weight daily.

ACKNOWLEDGMENTS

The authors wish to acknowledge the assistance of Mr. J. P. Jones, a graduate student in physiology, in the adaptation of analytical methods to the determination of calcium in the biological materials secured in this investigation.

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A STUDY OF TEST DOSE EXCRETION OF FIVE B COMPLEX VITAMINS IN MAN [1]

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(Received for publication June 7, 1951)

Among the many biochemical procedures which have been proposed to aid in evaluation of nutritional status in man is measurement of the urinary excretion of a vitamin or its metabolites following an oral or parenteral test dose of the vitamin (National Research Council, '49). It has been shown that subjects whose tissues are depleted in respect to a given vitamin tend to retain more of this nutrient than do normal subjects. The findings in this type of study depend in part upon the size of the test dose, the route of administration, and the duration of urine collection.

In the present investigation, an oral test dose of thiamine, riboflavin, and nicotinamide was administered to 115 individuals, including 39 essentially normal persons and 76 subjects [2] of varying nutritional status. Excretion of the vitamins was studied in relation to clinical findings and to dietary histories. Pyridoxal and folic acid were included in the test dose later in the investigation, in an attempt to determine whether there was any relationship between the level of excretion of these vitamins and nutritional status.

[1] This study was supported by grants from the Nutrition Foundation, Inc., the Williams-Waterman Fund of the Research Corporation, and the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

[2] These subjects were patients in the clinics or wards of the Charity Hospital of Louisiana, or in the Hutchinson Memorial Clinic of Tulane University.

EXPERIMENTAL

After preliminary experiments on several control subjects, the following test dose procedure was adopted: a tablet [3] containing approximately 5 mg of thiamine hydrochloride, 5 mg of riboflavin, and 50 mg of nicotinamide, was administered with two glasses of water in the morning, approximately half an hour before breakfast. In the latter part of this investigation, 25 mg of pyridoxal hydrochloride [4] (dissolved in part of the water) and a 5-mg tablet of folic acid [5] were given at the same time. Urine was collected for a 4-hour period, acidified with about 3 ml of glacial acetic acid, and kept in amber bottles in a refrigerator.

Analyses for the vitamins or their metabolites were performed by the following methods, some of which were modified for use in this laboratory: microbiological assays for thiamine (Sarett and Cheldelin, '44), riboflavin (Snell and Strong, '39), and folic acid (Mitchell and Snell, '41); fluorometric measurements for N^1-methylnicotinamide (N^1-Me; Huff and Perlzweig, '47) and 4-pyridoxic acid (Huff and Perlzweig, '44; Sarett, '51). For a short time early in this study other methods were used in the determination of thiamine (Mason and Williams, '42), riboflavin (Ferrebee, '40) and N^1-Me (Huff and Perlzweig, '43). The findings with the older methods were comparable to those obtained with the newer procedures and are included in this paper. Creatinine was measured photometrically in all urines, using an alkaline picrate reagent.

The volumes of the 4-hour specimen and the creatinine excretions were used as indices of the completeness of urine collection. A test was discarded when the urine volume was

[3] These tablets were prepared for this study by the Medical Research Department, Winthrop-Stearns Chemical Company, Inc., through the courtesy of Mr. Kenneth Smoot. They were found by analysis to contain approximately 5.2 mg thiamine, 5.5 mg riboflavin, and 56 mg nicotinamide per tablet.

[4] Pyridoxal hydrochloride was supplied through the courtesy of Merck and Company, Inc.

[5] Folic acid tablets were provided through the courtesy of the Lederle Laboratories Division, American Cyanamid Company.

less than 150 ml and the creatinine excretion was below the level expected for that individual, either on the basis of sex and weight or of his own 24-hour creatinine excretion, when available. Only two tests had to be discarded.

In preliminary experiments, urine was collected for two 2-hour periods following administration of the test dose of thiamine, riboflavin and nicotinamide, and for the subsequent 20-hour period. It was found that the portion of the test dose excreted in the first 2-hour period was small and the findings appeared to be influenced by variations in rate of absorption, in renal function, and in urine volume. The excretion in the first 4 hours, however, was found to represent over 50% of the extra excretion of each vitamin for the 24-hour period following administration of the test dose, and did not appear to be unduly affected by the aforementioned factors. The size of the test dose employed was large enough so that the total excretion in 4 hours was not markedly affected by the basal level of excretion. On the basis of these findings, this 4-hour test dose procedure was used throughout the study. Similar techniques have been found to be satisfactory in other laboratories (Johnson et al., '45; Oldham, Davis and Roberts, '46).

When folic acid (5 mg) and pyridoxal hydrochloride (25 mg) were incorporated into the test dose, similar criteria were used in choosing the amounts to be administered (Sarett, '51).

The 115 subjects were grouped in the following 6 categories for purposes of comparison and statistical evaulation of findings: (1) control subjects; (2) miscellaneous hospital patients; (3) subjects on weighed diets low in B complex vitamins; (4) patients with clinical evidence of vitamin B complex deficiency; (5) patients with clinical evidence of thiamine deficiency; and (6) patients with diabetes mellitus.

Dietary histories were obtained from 86 of the 115 subjects and the diets described were evaluated semi-quantitatively for content of vitamins of the B complex.[6] One point

[6] We are indebted to Miss Janis Gibbens for obtaining and evaluating the dietary histories.

was awarded for each daily serving of a food relatively high in these vitamins. Diets which supplied a total of 6 points and included at least three different foods which were good sources of B vitamins were classified "good." Diets which furnished three to 5 points from more than two food sources, or more than 5 points from only two food sources, were classified "fair." Diets which failed to meet these specifications were rated "poor."

RESULTS AND DISCUSSION

The excretion of vitamins following test doses varied considerably in some normal subjects who received more than one test and among individual members of each group. Although the range of findings was great, significant differences in vitamin excretion were observed in the several groups studied, as compared to the control group. The findings for each group are presented in table 1.

1. Control subjects

This group of 39 individuals received 52 tests with thiamine and riboflavin, 51 with nicotinamide, 44 with folic acid and 38 with pyridoxal. The subjects included doctors, employees in the medical school, and 10 individuals who were seen for routine examination in the Cancer Detection Clinic and found to be in good health. Findings with respect to these 10 individuals were similar to those for the rest of the control subjects. The test of one normal subject was excluded because both the urine volume and creatinine excretion fell below the limits previously designated.

The mean 4-hour excretion of vitamins or their metabolites by the control subjects following administration of the test dose is shown in table 1. No correlation was found between the age of the subjects, which ranged from 23 to 79 years, and excretion of any of the vitamins. Urine volumes were not significantly related to the level of test dose excretion.

TABLE 1

Four-hour urinary excretion following an oral test dose of B vitamins[1]

GROUP	THIAMINE		RIBOFLAVIN		N^1-METHYL-NICOTINAMIDE		FOLIC ACID		4-PYRIDOXIC ACID	
	Mean ± S.E.[2]	P[3]	Mean ± S.E.	P	Mean ± S.E.	P	Mean ± S.E.	P	Mean ± S.E.	P
	μg		*μg*		*mg*		*μg*		*mg*	
1. Control subjects	214 ± 18 (52)[4]	..	1,850 ± 98 (52)	..	8.0 ± 0.4 (51)	..	742 ± 60 (44)	..	9.5 ± 0.4 (38)	..
2. Miscellaneous hospital patients	188 ± 25 (14)		1,782 ± 115 (14)		5.0 ± 0.4 (14)	< 0.001	555 ± 150 (11)		9.0 ± 1.2 (6)	
3. Subjects on weighed diets low in B complex vitamins	49 ± 14 (12)	< 0.001	1,195 ± 88 (12)	< 0.001	6.8 ± 1.1 (5)		1,526 ± 300 (5)	< 0.001	10.4 ± 0.4 (3)	
4. Patients with signs of B complex deficiency	169 ± 26 (31)		1,985 ± 160 (30)		5.3 ± 0.4 (31)	< 0.001	486 ± 70 (24)	< 0.01	8.6 ± 0.6 (20)	
5. Patients with signs of thiamine deficiency	53 ± 12 (9)	< 0.001	1,505 ± 176 (9)		3.6 ± 0.5 (9)	< 0.001	385 ± 126 (6)	< 0.05	7.7 ± 1.2 (5)	
6. Patients with diabetes mellitus	437 ± 63 (23)	< 0.001	2,630 ± 117 (22)	< 0.001	4.5 ± 0.3 (23)	< 0.001	707 ± 70 (23)		10.0 ± 0.4 (22)	

[1] The test dose included 5 mg thiamine hydrochloride, 5 mg riboflavin, 50 mg nicotinamide, 5 mg folic acid and 25 mg pyridoxal hydrochloride.
[2] Standard error of the mean.
[3] P represents the probability of chance occurrence of the difference observed between test and control groups, calculated by use of Student's "t" test. Probability is shown only where difference was considered significant.
[4] Figures in parenthesis indciate number of tests performed.

The dietary histories of the control group indicated a higher intake of B complex vitamins than was observed in any other group with the possible exception of the subjects with diabetes mellitus (table 2). The economic level of the control subjects was superior to that of the members of other groups.

TABLE 2

Evaluation of diets as sources of B complex vitamins

GROUPS	NO. OF DIET HISTORIES	APPRAISAL		
		"Good"	"Fair"	"Poor"
		%	%	%
1. Control subjects	33	76	21	3
2. Miscellaneous hospital patients	11	27	55	18
3. Subjects on weighed diets low in B complex vitamins	12	0	0	100
4. Patients with signs of B complex deficiency	20	35	35	30
5. Patients with signs of thiamine deficiency	9	11	44	44
6. Patients with diabetes mellitus	22	68	23	9

2. Miscellaneous hospital patients

Tests were carried out on 12 subjects who were hospitalized for various medical conditions apparently not etiologically related to nutrition. The patients were all ambulatory and showed no clinical evidence of vitamin B complex deficiency.

The mean excretion of thiamine, riboflavin, and 4-pyridoxic acid in this group of patients was essentially the same as that of the control group (table 1). The mean folic acid excretion was lower than in the control group but the difference was not statistically significant. The mean excretion of N^1-Me by these patients was significantly lower than the excretion by the control subjects.

Dietary evaluation showed a poor intake of B vitamins (table 2) and of protein by these patients. This may explain the low excretion of N^1-Me, since niacin stores in the body depend on both the niacin and tryptophan content of the diet (Sarett and Goldsmith, '49). Other investigators have noted similar differences in the excretion of niacin metabolites between control subjects and miscellaneous hospital patients (Perlzweig et al., '42; Ruffin et al., '44; Goldsmith, '44).

3. Subjects on weighed diets low in B complex vitamins

This group of 12 essentially normal subjects was maintained in a metabolic ward on special weighed diets, low in protein and B complex vitamins, for from two and one-half to 6 weeks (average, 4.5 weeks) prior to the excretion tests. The diets provided 40 to 45 gm of protein, 0.5 to 0.8 mg of thiamine, 0.4 to 0.6 mg of riboflavin, 4.5 to 6.0 mg of niacin, and sufficient calories to maintain body weight.

The mean test dose excretion of thiamine and riboflavin by subjects in this group was significantly lower than that found in the control group (table 1). The excretion of N^1-Me was tested in only 5 subjects and while there was some diminution of the mean excretion as compared to the controls, the decrease was not statistically significant. The excretion of 4-pyridoxic acid was essentially the same as in the control group.

The marked and significant increase in folic acid excretion observed in the 5 subjects tested, as compared to the controls, is diametrically opposed to the significant decrease found in subjects with signs of vitamin B complex deficiency (table 1). The explanation of these findings is uncertain in view of limited knowledge of folic acid requirements and metabolism.

4. Patients with clinical evidence of vitamin B complex deficiency

Each of the 30 subjects in this group showed one or more signs commonly associated with deficiency of vitamins of the

B complex: cheilosis, angular stomatitis, glossitis, peripheral neuritis, or evidence of beri-beri heart disease. Among these patients were two with non-tropical sprue and one with nutritional macrocytic anemia. The dietary histories for this group of subjects (table 2) showed poor intake of B vitamins and were similar to those obtained for the group of miscellaneous hospital patients.

The test dose excretion of thiamine and of 4-pyridoxic acid in this group was somewhat lower than that in the control group but the decrease was not statistically significant (table 1). The excretion of riboflavin was similar to that found in the control subjects. The findings for N^1-Me were significantly lower than those of the control group, and comparable to those found in the miscellaneous hospital patients. Similar results have been reported previously in deficient subjects and hospital patients (Perlzweig et al., '42; Ruffin et al., '44; Goldsmith, '44; and Goldsmith et al., '50).

A significant decrease in folic acid excretion was observed in subjects with signs of vitamin B complex deficiency as compared to controls. This observation may suggest the presence of folic acid deficiency in some of these subjects. Further investigation of folic acid metabolism is necessary to interpret these results.

5. Patients with clinical evidence of thiamine deficiency

Signs of peripheral neuritis or beri-beri heart disease were the principle manifestations of vitamin B complex deficiency in 8 of the subjects in the preceding group. The excretion findings for these patients are presented separately in table 1. One of the subjects showed evidence of thiamine deficiency on two occasions and vitamin excretion was studied each time.

The mean excretion of the test dose of thiamine in this group was approximately the same as that found in subjects maintained on weighed diets low in B complex vitamins, and was significantly lower than that of the control group or

than that of the entire group of patients with signs of vitamin B complex deficiency (table 1). The mean test dose excretion of each of the other B vitamins by subjects with thiamine deficiency was also less than that found in the controls or in the entire group showing signs of vitamin B complex deficiency. The mean excretion of N^1-Me and folic acid was significantly less than that of the controls. The mean excretion of 4-pyridoxic acid was lower than that found in any other group but did not differ significantly from that of the control subjects.

6. Patients with diabetes mellitus

Tests were carried out on 22 patients with diabetes mellitus of varying severity. Although the diets of these subjects were approximately as good in sources of B complex vitamins as were those of the controls, 11 of the patients had clinical evidence suggestive of vitamin complex deficiency. No difference in the mean test dose excretion was observed between patients with and without signs of deficiency. There was no correlation between the test dose excretion of any of the vitamins and the duration, severity, or degree of control of diabetes, nor with recent change in weight. The findings are accordingly presented as being for a single group (table 1).

The mean excretion of thiamine, 437 μg, and of riboflavin, 2,630 μg, by the patients with diabetes mellitus was significantly higher than that found in the control subjects. The volume of urine excreted in 4 hours in the patients with diabetes was higher than in the controls. In the diabetic subjects there was a slight correlation ($r = +0.57$) between urine volume and riboflavin excretion and no correlation between urine volume and thiamine excretion.

Excretion of N^1-Me was significantly lower in patients with diabetes than in controls and was similar to that found in subjects with vitamin B complex deficiency. Excretion of folic acid and 4-pyridoxic acid was comparable to that observed in the controls.

Angular stomatitis and various types of glossitis are frequently seen in patients with diabetes mellitus. The findings relative to N^1-Me excretion suggest that some vitamin B deficiency may have existed in these subjects. In contrast to these findings, excretion of thiamine and riboflavin was unusually high. These data cannot be explained on the basis of dietary histories or other available information, and may indicate an impairment or abnormality in the utilization of thiamine and riboflavin in subjects with diabetes mellitus. Further studies are contemplated in connection with the utilization of B complex vitamins by the diabetic individual.

SUMMARY

An oral test dose of thiamine, riboflavin, nicotinamide, folic acid and pyridoxal was administered to 115 subjects of varying nutritional status, and the urinary excretion of these vitamins or their metabolites in the subsequent 4 hours was determined. Findings were evaulated in relation to clinical status and dietary histories for the following groups of subjects: (1) controls (essentially normal persons); (2) miscellaneous hospital patients; (3) subjects on weighed diets low in B complex vitamins; (4) patients with signs of vitamin B complex deficiency; (5) patients with clinical evidence of thiamine deficiency; and (6) patients with diabetes mellitus. The diets of the control subjects and of subjects with diabetes mellitus were much more satisfactory as to content of B vitamins that those of the other groups.

The mean thiamine excretion after the test dose was found to be significantly lower in patients with thiamine deficiency and in subjects who had been maintained on weighed diets low in B vitamins than in control subjects. Riboflavin excretion was significantly decreased only in the group of subjects on weighed diets low in B vitamins. Neither thiamine nor riboflavin excretion was significantly diminished in patients with clinical evidence of vitamin B complex deficiency.

The excretion of N^1-methylnicotinamide was less than that of the control subjects in all groups tested. The difference

was statistically significant in the miscellaneous hospital patients, in patients with signs of thiamine or vitamin B complex deficiency, and in subjects with diabetes mellitus.

Folic acid excretion was significantly lower in subjects with evidence of thiamine or vitamin B complex deficiency than in control subjects. In contrast to this, folic acid excretion in 5 subjects maintained on weighed diets low in B vitamins was significantly higher than that found in the controls.

The excretion of 4-pyridoxic acid was essentially the same in all of the groups tested.

In subjects with diabetes mellitus, the mean excretion of thiamine and riboflavin was significantly higher than that found in control subjects, while that of N^1-methylnicotinamide was significantly lower. These findings suggest a possible abnormality in the utilization of thiamine and riboflavin in patients with diabetes mellitus.

The test dose procedure described in this study appears to be useful in evaluating the nutriture of groups of individuals in regard to thiamine and niacin and to be of less value with respect to riboflavin, folic acid and pyridoxal.

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THE NUTRITION OF THE MOUSE

XI. RESPONSE OF FOUR STRAINS TO DIETS DIFFERING IN FAT CONTENT [1]

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TWO FIGURES

(Received for publication June 6, 1951)

In the course of earlier studies it was noted occasionally that mice of certain strains deposited excessive quantities of body fat when fed complete, highly purified diets. In view of the prominent role of obesity in the etiology of various diseases, it was decided to explore the possibility of inducing obesity by nutritional means alone. Obesity has been induced in experimental animals by means of electrolytic lesions of the hypothalamus (Brobeck, '46), by the feeding of gold-thioglucose (Brecher and Waxler, '49), by restricting activity (Ingle, '49) and so forth. The consistent production of obesity by nutritional means alone would indeed furnish a useful tool for studying the role of obesity in cardiovascular diseases, hormonal disorders and cancer.

METHODS

Male mice of 4 strains (C_{57}, I, C_3H and A) were weaned when 21 days old and immediately placed on one of the experimental diets (table 1). These animals were offspring of the colonies maintained in this laboratory. All 4 strains were received from Dr. L. C. Strong, Yale University, in 1946 and

[1] Supported by grants from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council; the National Cancer Institute, U. S. Public Health Service; and the Anna Fuller Fund.

have been maintained by brother-sister matings. Throughout the experimental period food and water were supplied ad libitum. The food consumption of 43 animals was measured by means of a special wire-screen device which reduced food scattering to negligible proportions without interfering with the accessibility of the food. Water consumption was measured in some cases. All experimental animals were maintained in individual screen-bottom cages.

TABLE 1

Composition of diets

CONSTITUENTS	DIET NUMBER					
	223	224	225	226	227	245
Casein [1]	300	900	450	300	300	300
Dextrin	600	..	..	..	500	400
Sucrose	..	..	..	175	..	..
Salt mixture A [1]	50	50	75	50	50	50
Corn oil [1]	50	50	75	50	50	50
Hydrogenated cottonseed oil	..	..	400	425	100	200

[1] Water-soluble B vitamins were dried on the casein, and the fat-soluble vitamins dissolved in the corn oil in the amounts described by Fenton and Carr ('51). The composition of the salt mixture is also given in the same paper. The vitamin content of diet 225 was 50% greater than that of the other diets.

In order to test the effect of dietary composition on gain in body weight in the adult animal, a group of A strain males and females which had been raised to maturity on a commercial stock ration were placed on diets 224, 225 and 227 (table 1). The animals were selected as uniformly as possible with respect to sex and initial body weight. They were 12 to 13 months old. A total of 228 mice were used in these studies.

RESULTS

Growth curves of the 4 strains of mice fed diets containing different levels of fat are shown in figure 1. At the 5% fat level C_3H mice showed the best weight gains. At higher fat levels there was little difference in the total weight gained

by C_3H and A strain mice. The weight gain of C_{57} mice was nearly identical with that of the A strain animals when fed the 5% fat diet. However, when higher fat levels were used,

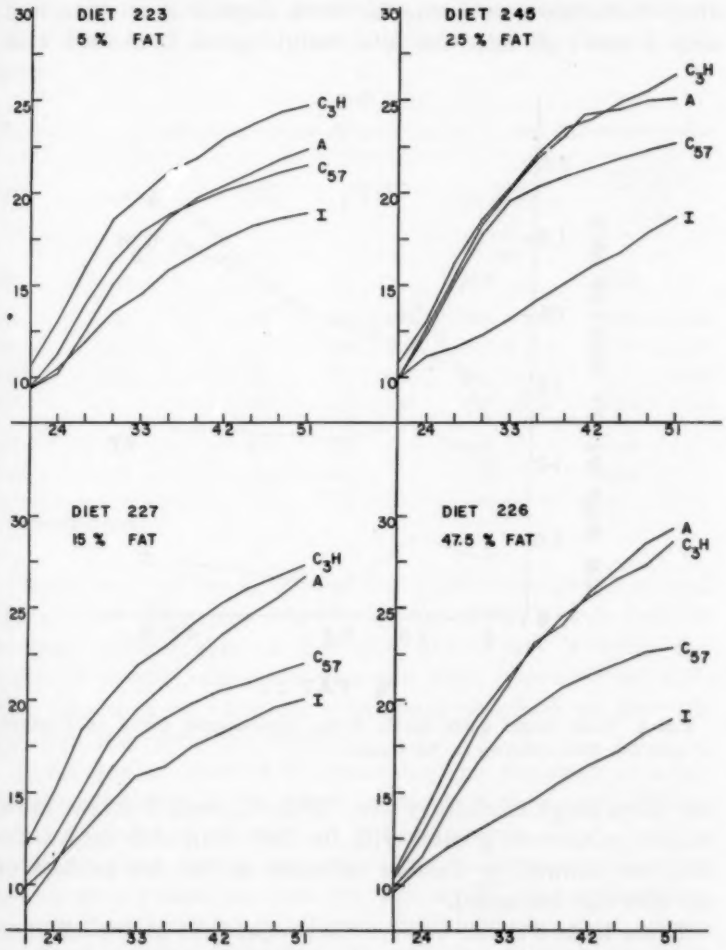

Fig. 1 Weight (in grams) of 4 strains of mice on diets of different fat content plotted against age in days.

the weight gains of the A strain mice were distinctly greater than those of the C_{57} mice.

In figure 2 the total weight gains during the 30-day experimental period are plotted against the percentage of fat in the diet. While the results are far from regular it is clear that with A and C_3H mice the total weight gains increased with

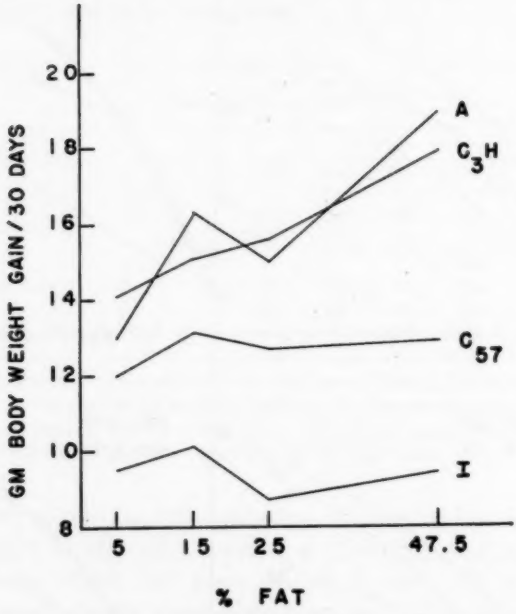

Fig. 2 Total weight gains during 30-day experimental period of 4 strains of mice fed diets differing in fat content.

the percentage of dietary fat. With C_{57} and I strain mice, weight gains were greater with the 15% than with the 5% fat diet but showed no further increase as the fat content of the diet was increased.

From table 2 it can be seen that regardless of the effect of dietary fat level on growth, the efficiency of food utilization for growth increased with the fat content of the ration.

Comparison of the C_3H and I strains at a fat level of 47.5% shows the former's great efficiency of food utilization for growth. Comparison of all 4 strains at the 15% fat level shows the A strain to be least efficient in food utilization and the C_{57} the most efficient. Some data have been compiled which show that later in life these relations may no longer hold true.

TABLE 2

Efficiency of food utilization for weight gain during 15 days following weaning

DIET NO.	STRAIN	FAT LEVEL	WEIGHT GAIN	FOOD CONSUMPTION	WEIGHT GAIN PER GRAM OF FOOD CONSUMED
		%	gm	gm	gm
223	I	5	4.4	32.6	0.135
227	I	15	8.2 ± 0.38 [1]	36.5 ± 0.63	0.224 ± 0.008
227	A	15	8.6 ± 0.46	41.9 ± 1.16	0.206 ± 0.016
227	C_3H	15	10.5 ± 0.31	48.3 ± 0.65	0.221 ± 0.004
227	C_{57}	15	10.0 ± 0.27	37.3 ± 0.62	0.269 ± 0.008
226	I	47.5	5.9	24.2	0.244
226	C_3H	47.5	10.5	31.6	0.334

[1] Standard error.

Since mice fed the 47.5% fat diet (226) consumed less food (in grams) than mice fed the low-fat diets, it was decided to study weight gain on diet 225 (47.5% fat) in which the levels of protein, salts and vitamins were increased by 50%. The results were identical with those obtained on diet 226 and are therefore not reported.

It seemed of interest to determine the response of adult mice to: (a) a synthetic diet of relatively normal composition, (b) a high-fat diet, and (c) a high-protein diet. To this end adult mice, raised and maintained on a commercial stock ration, were placed on diets 224, 225 or 227. The animals receiving the high-protein diet at first lost and then slowly regained weight. After 8 weeks on this ration the average body weight was still considerably below that at the beginning

of the synthetic diet feeding. Mice fed the relatively normal synthetic diet lost some weight at the beginning of the experiment, but rather quickly regained it. After 8 weeks the average weight was slightly above that observed at the beginning of the period. The group of animals receiving the high-fat diet lost very little weight at the start of the synthetic diet feeding. They quickly regained the lost weight and continued to gain at a significant rate. After 8 weeks on this high-fat diet the average body weight was roughly 10% above the starting weight.

DISCUSSION

Forbes et al. ('46), Deuel et al. ('47) and others have shown that fat in excess of the quantity necessary to supply the essential fatty acids has a beneficial effect on growth. Bosshardt et al. ('50) have shown that increasing the fat content of the ration in the absence of vitamin B_{12} resulted in increasing the growth rate, the maximum effect being noted with a 40% fat diet. Higher levels of fat depressed the growth rates. In the presence of vitamin B_{12} maximum growth rates were noted at all fat levels up to 40%. The results of our experiments indicate, on the other hand, that the effect of the fat content of the ration upon weight gain depends most decidedly upon the strain of animal employed in such investigations. As figure 2 shows, mice of the A and C_3H strains very clearly increased the rate of weight gain with increasing fat content in the ration. On the other hand, mice of the C_{57} and I strains showed no further increases in the rates of weight gain when the fat content of the diet was increased above 15%.

The differences in growth response of different strains of mice to increasing dietary fat levels could be explained in several ways. It is conceivable that C_{57} and I strain mice have a lower requirement for vitamin B_{12} than mice of the A and C_3H strains (as is the case for riboflavin and pantothenic acid; Fenton and Cowgill, '47; Fenton et al., '50). If such a small requirement of vitamin B_{12} could be met by

existing body stores and by the ingestion of the small quantities present as impurities in the ration, the C_{57} and I strain mice would be comparable to those animals of Bosshardt which had vitamin B_{12} administered and showed maximum growth at all fat levels up to 40%. Another possibility is that mice of the C_{57} and I strains more efficiently adapt their caloric intake to their caloric requirement in the face of diets of differing caloric densities.

Account must be taken of the fact that the diets containing 5, 15 and 25% fat contained dextrin as the carbohydrate. On the other hand, the 47.5% fat diet contained sucrose. The latter was originally designed for quite another experiment but the results obtained with it are included here because they supplement the experiments here reported. Some experiments with high-fat, dextrin-containing diets have indicated little difference between them and sucrose-containing diets such as 226.

Since considerable variation in weight gain was encountered with I strain animals, several groups of these mice were studied at each dietary fat level. The results obtained with these several groups were similar but by no means identical. No explanation can be offered at the present time for this high degree of variability observed in mice of the I strain.

Casual observation suggested that mice of different strains might be consuming substantially different quantities of water. Careful examination of this point, however, revealed no significant differences.

Mice were continued on the various experimental rations for 5 months following the initial 30-day experimental period. During this time animals of the C_3H and A strains accumulated considerable body weight, much of this in the form of fat as shown by carcass analysis. On the other hand, C_{57} and I strain animals gained little weight following the initial 30-day period. There was a certain degree of correlation between the weaning weight of the mouse and the final total body weight. C_3H and A strain mice of greater

than average weaning weight showed a greater tendency to become obese than animals of less than average weaning weight.

SUMMARY AND CONCLUSIONS

1. Increasing the fat content of the diet increased the weight gain of C_3H and A strain mice but not of C_{57} and I strain animals.
2. Efficiency of food utilization for body weight gain increased with increasing dietary fat levels.
3. Transferring mature animals from a commercial stock ration to a highly purified diet caused least disturbance in body weight if the synthetic diet was of the high-fat type.

ACKNOWLEDGMENTS

We wish to thank Dr. E. L. Sevringhaus of Hoffman-La-Roche, Inc., for generous gifts of many of the B vitamins used in this study. The folic acid was supplied by Dr. T. H. Jukes of the Lederle Laboratories.

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LEUCINE AND HISTIDINE TOLERANCE IN THE HUMAN [1]

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TWO FIGURES

(Received for publication May 21, 1951)

Various workers have been unable to show any direct relationship between the urinary excretion of most of the amino acids and their dietary intake (Woodson, Hier, Solomon and Bergeim, '48; Steele, Reynolds and Baumann, '50). Neither do the quantitative relationships of the amino acids to each other as found in the urine reflect the pattern found in the blood (Harper, '49; Steele et al., '50). The purpose of this study was to learn more about the relationship among the amino acids in the blood and in the urine. Leucine and histidine were the amino acids chosen for this purpose.

EXPERIMENTAL

Subjects

Six women and one man served as subjects. All were adult college students in good health, and free from known metabolic disorders. Three of the subjects were fed amounts of L-leucine ranging from 1.5 to 10 gm dissolved in 500 ml of water. The other 4 subjects ingested 1.5 and 5 gm of L-histidine (given as the hydrochloride) dissolved in 500 ml of water.

[1] Presented in part before the American Institute of Nutrition, Federation of American Societies for Experimental Biology meeting, Cleveland, 1951.

The assistance of Marie L. Skellinger in part of the work is gratefully acknowledged.

Experimental plan

The subjects fasted over-night, usually for a period of 8 to 10 hours. In the morning the subjects reported to the laboratory in a fasting state and 10 ml of venous blood were withdrawn, after which they ingested a weighed amount of the L-isomer of the appropriate amino acid. Venous blood was withdrawn one-half, one, one and one-half, ocasionally two and one-half, and 4 hours after ingestion of the amino acid solution. Three urine collections were made. The first, the morning voiding, represented the fasting period. The second period included urine formed up to 60 min. after the ingestion of the amino acid, and the third period, that urine formed 61 to 240 min. after ingestion of the solution. These collections were analyzed separately for their amino acid content.

Preparation of samples for analysis

Heparin was used as an anti-coagulant for the blood. Plasma and formed elements were separated by centrifugation. The white cells were separated from the red cells and discarded. The red cells were washed twice with physiological saline to remove any residual plasma. The method of Hier and Bergeim ('45) was used to make tungstic-acid, protein-free filtrates of known volumes of the plasma and of red blood cells.

Aliquots of the urine were acid-hydrolyzed by the method of Steele et al. ('50).

Analytical methods

Microbiological methods were used for determination of the amino acids in the free form in the filtrates from the plasma, the red blood cells and in the acid-hydrolyzed urine (Steele et al., '49). Determination of the urea in the plasma was made by the Karr method (Karr, '24) adapted to the Evelyn colorimeter.

Calculation of renal clearance

The formula $\frac{uv}{p}$, where U = milligrams per cent of amino acid in the urine, V = milliliters of urine excreted/minute,

and P = milligrams per cent of amino acid in the plasma, was used. P was relatively constant during the collection of urine for the fasting clearances. To calculate the hour clearances, the P value at one-half hour was used. As this value was changing during the urine collection time employed, these clearances should be regarded as approximate only and in most cases probably represent the minimum value.

TABLE 1

Effect of ingestion of different amounts of L-*leucine on the plasma content, urinary excretion, and renal clearance of leucine*

SUBJECT	C.H. (♀)		C.L. (♀)			H.B. (♂)		
GM LEUCINE INGESTED	1.5	5.0	1.5	5.0	10.0	5.0	7.5	10.0
time, min.			*Plasma levels (μg/ml)*					
0	19	20	20	17	17	25	25	25
30	67	153	52	162	141	126	194	185
60	40	91	40	90	210	91	132	175
90	28	68	32	39	189	58	87	131
240	20	27	19	25	40	26	35	33
			Urine levels (mg/hour)					
Over-night fast	0.6	0.5	0.6	0.6	0.6	1.0	1.5	1.2
0–60 min. post-ingestion	1.9	2.3	1.7	2.0	2.8	1.2	2.0	2.7
61–240 min. post-ingestion	0.7	0.8	0.9	1.0	1.3	0.9	1.0	1.0
Percentage dose recovered	0.1	0.05	0.1	0.05	0.04	0.0	0.0	0.01
			Leucine clearance					
Fasting	0.5	0.4	0.5	0.6	0.5	0.7	1.0	0.8
One-hour	0.5	0.3	0.5	0.3	0.3	0.5	0.5	0.2

RESULTS

Leucine experiment

Three subjects, two females and one male, ingested the leucine solutions (table 1). The doses employed always caused an increase in the plasma leucine; the peak value came at 30 min., when 1.5 or 5 gm of leucine were given. When the dose was increased to 7.5 or 10 gm, an elevation of plasma

leucine lasting from 30 to 90 min. occurred. Although the leucine content of plasma was greatly elevated, very little of the leucine was excreted into the urine. When subject C.H. was fed 1.5 gm of L-leucine, the plasma at 30 min. contained 67 μg/ml of leucine, in contrast to 19 μg/ml at fasting, and the leucine content of urine increased from 0.6 mg/hour at fasting to

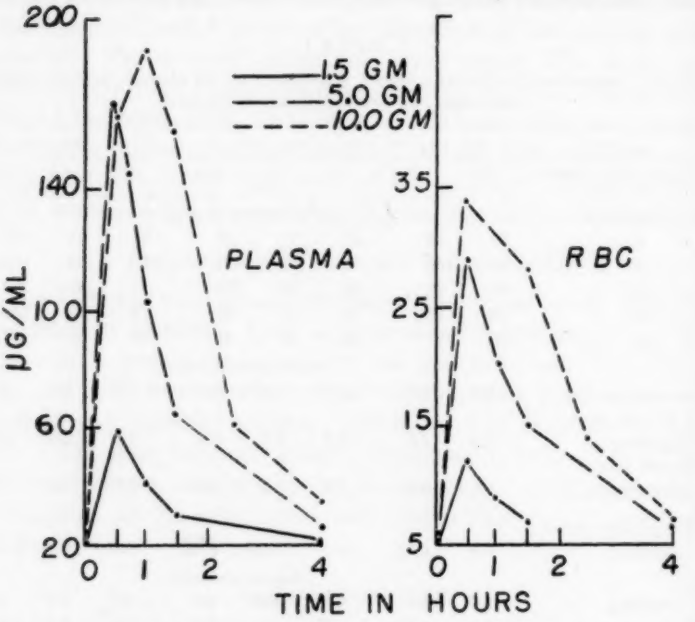

Fig. 1 Average leucine content of plasma and red blood cells over a period of 4 hours resulting from the ingestion of 1.5, 5.0, and 10.0 gm of L-leucine.

1.9 mg/hour for the one-hour collection period. If 5 gm of leucine were given, the plasma was elevated to 153 μg/ml at 30 min., but the urinary excretion of leucine increased to only 2.3 mg/hour. These elevations are typical of those found with all three subjects. In no case was there a proportional increase between the one-half hour plasma and one-hour urine values. The amount of leucine recovered in the urine over

a 4-hour period never exceeded 0.1%. This percentage recovery tended to fall when a higher dose of leucine was ingested.

Renal clearances of leucine ranged from 0.2 ml to 1.0 ml (table 1). Clearance values at one hour were usually lower than those found during the fasting state. The two subjects, C.H. and C.L., who were fed 1.5 gm of leucine had the same fasting and one-hour clearance values.

TABLE 2

Effect of ingestion of different amounts of L-*histidine on the plasma content, urinary excretion, and renal clearance of histidine*

SUBJECT	D.A. (♀)		M.M. (♀)		B.E. (♀)		L.N. (♀)	
GM HISTIDINE INGESTED	1.5	5.0	1.5	5.0	1.5	5.0	1.5	5.0
time, min.				*Plasma levels (μg/ml)*				
0	15	19	15	10	20	21	14	16
30	40	123	35	117	51	147	112	63
60	36	115	31	132	54	144	31	123
90	20	145	22	144	15	108	20	147
240	18	34	18	45	15	51	13	35
				Urine levels (mg/hour)				
Over-night fast	7	9	8	5	9	5	11	5
0–60 min. post-ingestion	32	95	31	55	40	58	19	40
61–240 min. post-ingestion	8	28	12	45	14	49	10	36
Percentage dose recovered	1.9	2.7	2.3	3.2	3.1	3.8	0.3	2.5
				Histidine clearance				
Fasting	7	8	8	8	6	4	13	5
One-hour	13	13	14	8	5	7	3	10

Figure 1 shows graphically the plasma and red blood cell leucine levels resulting from the 1.5, 5, and 10 gm doses. There was a sharp rise in plasma and red blood cell leucine levels and then a rapid fall. The leucine content of the red blood cells was less than that of the plasma but the shapes of the curves are very similar.

Histidine experiment

Four female subjects were fed two levels of L-histidine, 1.5 and 5 gm (table 2). At one-half hour the 1.5 gm dose elevated plasma histidine two- to 8-fold over the fasting level, while the 5 gm dose elevated the level 7- to 11-fold. This histidine content remained elevated for a short time and then declined.

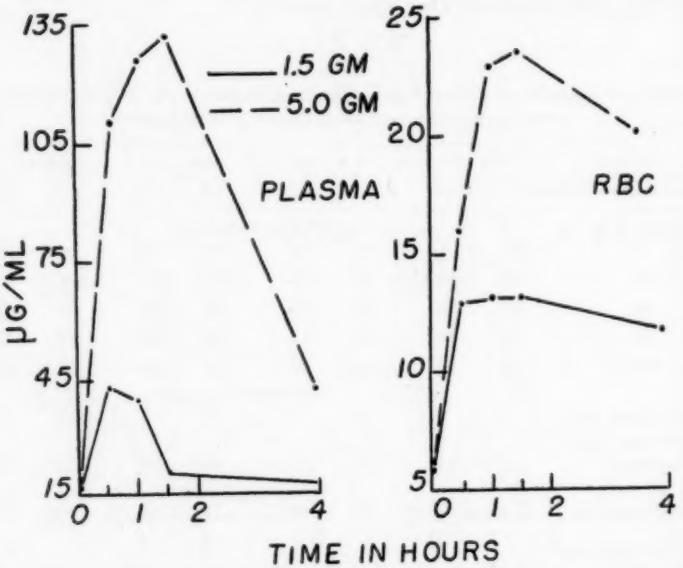

Fig. 2 Average histidine content of plasma and red blood cells over a period of 4 hours resulting from the ingestion of 1.5 and 5.0 gm of L-histidine.

Subject L.N., when given 1.5 gm of histidine, showed a sharp rise, then a rapid fall of plasma histidine. This was unlike the response of the other three subjects.

The increased histidine level of the plasma was always followed by an increased urinary excretion of histidine. When the plasma histidine increased from 15 μg to 40 μg/ml in D.A., the urinary excretion increased from 7 to 32 mg/hour. The percentage increase in the one-hour urine value when com-

pared with the fasting urine value was always greater than the percentage increase of the one-half-hour plasma histidine as compared to the fasting value, except for subject L.N. at the 1.5 gm dose.

The amount of the histidine dose recovered in the urine over the 4-hour period ranged from 0.3 to 3.8%. A greater percentage was recovered with the 5 gm dose than with the 1.5 gm.

The renal clearances for histidine ranged from 3 to 14 ml. The one-hour clearances were elevated over the fasting clearances except in three instances, M.M. at 5 gm, B.E. at 1.5 gm, and L.N. at 1.5 gm intakes.

Figure 2 shows the averages of the plasma and red blood cell histidine values for three of the subjects, D.A., M.M., and B.E. Values for subject L.N. were not included in these averages because of her atypical response to the 1.5 gm histidine dose. The plasma histidine remained elevated for a longer period of time than did the plasma leucine when comparable doses were ingested. The rate of fall of the red blood cell histidine was much slower than that of the plasma.

DISCUSSION

From the data presented there appear to be marked differences in the selectivity of the kidney tubules for the individual amino acids during normal reabsorption activity. This is in agreement with the work of Ussing ('45) and of Sheffner et al. ('48) on human subjects. Leucine was more efficiently reabsorbed at elevated plasma levels than was histidine. That the difference found is a result of kidney action and not of the actual amount of amino acid present in the blood seems evident from the data. In no instance was the elevation of plasma histidine as great as that of plasma leucine, yet a higher percentage of the histidine dose was recovered in the urine. Perhaps some of the increase in excretion could have been caused by the tendency of the plasma histidine to remain elevated for a longer period than the plasma leucine.

Although 10 gm of leucine caused a high plasma leucine content for a period of 90 min., the greatest amount of leucine recovered in the urine over 4 hours was 0.04% of the administered dose. The amount of histidine recovered in the urine increased as the administered dose of histidine and the plasma histidine increased, but the increase in urinary excretion was not directly proportional to the increase in dietary intake. Thus, while the histidine content of the blood affects the histidine content of the urine, there are apparently other mechanisms which prevent this from being a clear-cut relationship.

Only a small percentage of the leucine and histidine was recovered in the urine; thus, both amino acids must be picked up rapidly by the body cells.

The amino acid content of the circulating fluid must be higher than that of the cell if passage of the amino acid into the cell is dependent upon concentration only. Conversely, as the amino acid content of the circulating fluid falls, the amino acid should pass out of the cell unless the cell possesses active concentrating abilities or the amino acid is present in an unstable combination which does not pass through the cell wall. These two possibilities have been postulated by Van Slyke and Meyer ('13) and Christensen et al. ('48). In the present experiment the leucine content of the red blood cells paralleled that of plasma. Even though the leucine content of plasma was higher than that of the red blood cells, the microbiologically-available leucine in the cells declined after 30 min. If the leucine did not diffuse out of the cell, it must have been formed into a complex unavailable to *Leuconostoc mesenteroides* P-60.

The histidine content of the red blood cells paralleled the histidine content of the plasma in the initial rise but remained elevated as the plasma values fell. Prolonged elevation of the histidine content of the cell may be due to: (1) the surrounding fluid (plasma) contains more histidine than the cell, thus the amino acid did not diffuse out; (2) formation of

a combined form of histidine which is unable to pass through the cell wall. This altered form must be available for utilization by the microorganism.

In agreement with Hier ('47), who used dogs, high plasma levels were accompanied by a decrease in the plasma content of phenylalanine and valine in the human, while the elevation of plasma histidine did not affect leucine, methionine, or valine levels.

SUMMARY

Seven human subjects were fed various amounts of L-leucine or L-histidine dissolved in 500 ml of water. Blood and urine samples, collected for the fasting period and at intervals up to 4 hours after administration of the test solutions, were analyzed for amino acids by microbiological methods.

Leucine ingestion resulted in a rapid rise in plasma leucine, followed by a relatively rapid decline. The leucine content of the red blood cells paralleled the leucine content of the plasma. The amount of leucine recovered in the urine over a 4-hour period did not exceed 0.1%.

The ingestion of histidine caused the plasma histidine to rise, remain elevated for varying lengths of time, and then decline. The histidine content of the red blood cells became elevated but showed only a slight tendency to return to fasting levels during the 4 hours of the test period. The amount of histidine recovered in the urine ranged from 0.3 to 3.8%.

Renal clearances for leucine ranged from 0.2 to 1 ml; for histidine, from 3 to 14 ml.

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A STUDY OF HISTIDINE DEFICIENCY AND NITROGEN BALANCE UNDER AD LIBITUM AND FORCE-FEEDING CONDITIONS [1]

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ONE FIGURE

(Received for publication April 23, 1951)

During the last few years many reports have been published concerning the retention of nitrogen by animals fed an incomplete amino acid mixture and using the nitrogen balance technique of Mitchell ('24). Wolf and Corley ('39) reported histidine to be essential in maintaining nitrogen balance in adult female rats. Burroughs et al. ('40) found that protein-depleted adult female rats force-fed a histidine-deficient ration did not require histidine to maintain nitrogen equilibrium, while Wissler et al. ('48) observed a negative nitrogen balance with adult male rats force-fed a histidine-free ration. Denton, Williams and Elvehjem ('50) have suggested that these differences concerning nitrogen balance in rats fed an amino acid-deficient ration may possibly be due to differences in the level of nitrogen intake as well as the amino acid composition of the ration. These workers were able to maintain male weanling rats in positive nitrogen balance on a ration devoid of methionine by force-feeding a constant high level of nitrogen.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation; by funds from the Nutrition Foundation, Inc., New York, N. Y.; and by funds from the Robert Gould Research Foundation, Cincinnati, Ohio.

It has been noted (Wissler et al., '48; Frazier et al., '47) that there is a definite impairment of appetite in animals fed ad libitum a ration free of any one of the essential amino acids. Thus the variation of nitrogen intake and the effects of inanition complicate a study of nitrogen balance under ad libitum feeding conditions. When animals are force-fed, the effects of variable food intake due to variations in appetite can be negated.

The present work was undertaken in order to extend the results of Denton et al. ('50), which showed a remarkable correlation between nitrogen intake and nitrogen balance in rats given a methionine-deficient ration either ad libitum or by force-feeding procedures.

EXPERIMENTAL

Twenty-eight male, weanling rats of the Holtzman strain were divided at random into 4 groups: two groups of 8 animals each and two groups of 6 animals each. They were placed in metal metabolism cages and fed the purified ration of Ramasarma et al. ('49) for about a week or until a weight of 60 gm was reached. This ration consisted of a purified amino acid mix (16%), salts IV (Hegsted et al., '41), corn oil, the known vitamins and sucrose. During this pre-test period, daily food consumptions were noted in order to determine the amount of food to be fed to the group receiving the ration by stomach tube. All L-amino acids used in the ration were shown to be devoid of histidine by both microbiological and chemical assays.

The two groups of 6 animals each were fed ad libitum throughout the 14-day test period. One group received the complete ration and the other the complete ration less histidine. The other two groups, of 8 animals each, were force-fed the complete and deficient ration by stomach tube.

The pre-test feeding period was used to accustom the animals to the ration and bring them up to the desired weight level. This period also made it possible to accustom the force-fed group to the stomach tube procedure and the animals to

be fed ad libitum to the tongue-feeding procedure. The former group was fed increasing amounts by stomach tube each day until the desired level was reached. Thus diarrhea was almost completely prevented. In order to eliminate as much contamination of the urine and feces as possible, the animals fed ad libitum were tongue-fed through wire mesh (No. 4 mesh). The ration to be force-fed was made up in a slurry with water so that 7 ml of this would be equivalent to 6 gm of dry ration. The animals fed ad libitum consumed an average of 6 gm of ration (96.6 mg N) per day during the pretest period, and this level was chosen to be force-fed. At first, force-feedings were made three times daily at 5-hour intervals, but it was later found necessary to force-feed 4 times per day to give the deficient animals an opportunity to utilize more efficiently the food already in the gastrointestinal tract.

All urines were collected under toluene acidified with HCl. Collections were made every 4 days and in the case of the animals fed ad libitum urines from each two animals were pooled. The urines were then diluted to 250 ml for nitrogen determinations. Fecal collections were made at the same time as the urine collections. The feces were covered with 95% alcohol, acidified with one drop of concentrated HCl and dried in a steam oven. The dried samples were weighed and ground in a mortar, and portions were taken for nitrogen determination. A semi-micro Kjeldahl procedure was used for nitrogen determinations of the urine and feces samples.

RESULTS AND DISCUSSION

In order to facilitate correlation of the weight changes, nitrogen intake and nitrogen balance, the results of both the force-feeding and ad libitum feeding experiments are presented in graph form in figure 1 (curves I, II, III, IV, V, VI). The distribution of excretory nitrogen between the urine and feces is presented in table 1.

The weight curves in figure 1 (I and IV) indicate that there was an immediate weight gain in the animals receiving the complete ration either ad libitum or by force-feeding.

No lag was noted in either curve, since the animals had been fed the complete ration previous to the start of the experiment and were steadily gaining weight when put on test. The differences in weight gain between the animals receiving the

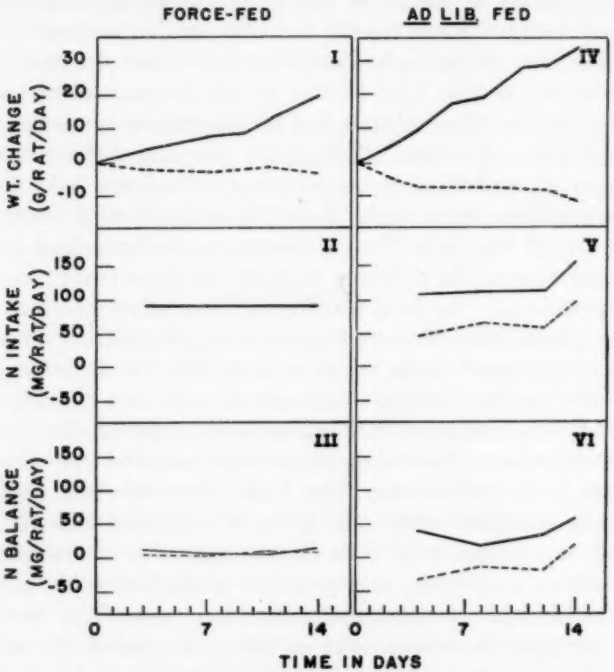

Fig. 1 Weight change, nitrogen intake, and nitrogen balance curves of animals receiving a complete or histidine-free ration ad libitum or by force-feeding.

– – – – = histidine-deficient animals
——— = control animals

complete ration ad libitum and by stomach tube may be attributable to the amount of food consumed, because as the animals fed ad libitum grew, they consumed more food. An immediate loss in weight was noted with the animals fed the histidine-free ration ad libitum, while there was little

loss of weight with the group receiving the same ration by stomach tube throughout the experiment. This difference in weight loss may again be attributable to differences in food consumption. These weight changes are similar to those observed by Denton et al. ('50) when feeding a ration free of methionine.

TABLE 1

Distribution of nitrogen output for animals fed a complete and histidine-free ration under ad libitum and force-feeding conditions

REGIMEN	PERIOD	NITROGEN OUTPUT	
		Urine	Feces
	days	*mg N/rat/day*	
Complete ration ad libitum	1–4	73.0	5.6
	4–8	90.0	7.5
	8–12	81.0	6.6
	12–14	92.0	5.1
Deficient ration ad libitum	1–4	73.6	4.2
	4–8	77.6	3.3
	8–12	78.1	4.3
	12–14	77.3	2.6
Complete ration force-fed	1–3	78.0	7.3
	3–7	86.2	8.5
	7–11	86.0	6.7
	11–14	75.2	5.5
Deficient ration force-fed	1–3	87.4	4.7
	3–7	89.2	6.9
	7–11	81.0	8.8
	11–14	72.0	10.3

The nitrogen intake for the two groups is presented in curves II and V. The nitrogen intake of the force-fed animals was constant with both the complete and deficient rations, while that of the animals fed ad libitum varied greatly. An immediate drop in food consumption was noted for the animals receiving the deficient ration ad libitum. This is in agreement with the findings of Frazier et al. ('47). However, the animals were apparently able to accustom themselves some-

what to this deficient ration, as was shown by an increase in food intake as the experiment continued.

Of particular note is the striking similarity between the nitrogen balance curves III and VI and the nitrogen intake curves II and V. The variations in nitrogen intake were generally closely followed by similar variations in nitrogen balance. When the intake was controlled, there was a similar and fairly constant nitrogen balance with both the complete and deficient rations. The overlapping of these two curves indicates that the level of nitrogen intake may exert a greater influence on the nitrogen balance than does the lack of histidine. This lends added support to the suggestion by Denton, Williams and Elvehjem ('50) that the level of nitrogen in the ration as well as the actual amount consumed must be considered when observing the effects on nitrogen retention of an amino acid deficiency. The discrepancy between our results and those reported by Wissler et al. ('48) for a histidine deficiency may be attributable to the different levels of nitrogen intake employed. They force-fed a level of nitrogen to adult (about 300-gm) rats which was lower in proportion to body weight than the level we fed in the present work to weanling (about 70-gm) rats.

It was very interesting to note the physical condition of the animals during the experiment. Those receiving the complete ration both ad libitum and by stomach tube remained in good health and gained weight throughout the experimental period. The animals force-fed the complete ration appeared grossly just as healthy as the animals fed the complete ration ad libitum. A striking difference, however, was noted between the appearance of the animals receiving the histidine-free diet by force-feeding and those receiving it ad libitum. At the end of the 14-day period, with the exception of weight loss, there was little apparent change in the animals fed ad libitum the ration devoid of histidine. They remained quite active and there were no deaths. In marked contrast, the animals force-fed the deficient ration soon (within 4 to 6 days) developed bloody mouth and paws, loss of hair, lack of coordina-

tion and a generally sluggish condition. Here, then, were two significantly different conditions in animals receiving the same ration by two different methods. This may possibly be attributed to the stomach-tube feeding, but the animals receiving the complete ration by exactly similar means showed no ill effects. The only other apparent difference was in the amount of food consumed. The force-fed deficient group was fed 6 gm of ration per day regardless of their condition or desire for food, and it was this group that exhibited the marked symptoms mentioned above. Animals in this group began to die after about 9 days and post mortems on each animal revealed a large amount of food in the stomach. The mortality rate was greater than 50%. Shortly before death the animals appeared quite lethargic, with almost complete lack of coordination in walking.

It will be noted in table 1 that there was a steady increase in the amount of fecal nitrogen for the group force-fed the deficient ration. Since this amount was not large in comparison to the total nitrogen output, it is difficult to say whether this increase was significant. It was, however, correlated with a decrease in urinary nitrogen. This suggests passage of a small amount of the food through the gastrointestinal tract without absorption.

Frazier et al. ('47) suggest a general metabolic disturbance as the factor responsible for the impairment of appetite. Perhaps certain proteins necessary for food utilization are not synthesized rapidly enough to handle the food intake. This could explain the lag of several days before onset of symptoms. Further studies on this subject are now in progress.

SUMMARY

The effects of a histidine deficiency on weight, nitrogen intake and nitrogen balance have been investigated, employing both ad libitum and force-feeding procedures.

The variation in the weights and nitrogen balances of the animals receiving the ration ad libitum and by a force-feed-

ing procedure may well be attributable to differences in the amounts of ration consumed.

A lack of food utilization in animals force-fed the amino acid-deficient ration was noted and a corresponding lack of appetite in animals fed the same ration ad libitum. A possible biological mechanism for explaining these observations has been suggested.

ACKNOWLEDGMENT

The authors wish to thank Mr. Donald J. Siehr for technical aid during the later phases of this work.

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RADIOPHOSPHORUS (P^{32}) AS TRACER FOR MEASURING ENDOGENOUS PHOSPHORUS IN COW'S FECES[1]

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ONE FIGURE

(Received for publication April 25, 1951)

INTRODUCTION

Fecal phosphorus may be partitioned into exogenous and endogenous phosphorus. The exogenous fecal phosphorus is undigested phosphorus from food. The endogenous phosphorus reaches the intestinal contents either by diffusion from blood or interstitial tissue fluid, as part of secretions such as saliva, as a component of cells or cell fragments sloughed off from the intestinal lining, or contained in phagocytes. The phosphorus which comes to the feces in microorganisms is either exogenous, namely that part which the microorganisms have taken up from undigested compounds in the intestinal tract, or endogenous, namely that part which the intestinal microorganisms get from digestive juices or other sources of endogenous phosphorus.

Knowledge of endogenous fecal phosphorus is important for determination of the "true" digestibility of food phosphorus, that is, the degree to which food phosphorus can be absorbed by the animal. The apparent digestibility

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$\frac{\text{food phosphorus} - \text{fecal phosphorus}}{\text{food phosphorus}}$, is a valid basis for estimating the "true" digestibility only when the endogenous phosphorus in the feces is a small part of the total fecal phosphorus.

Our trials show that the endogenous fecal phosphorus may exceed the undigested food phosphorus. Aside from estimation of true digestibility, the measurement of endogenous fecal phosphorus is important for knowledge of an animal's phosphorus metabolism. This is especially true for cattle, whose rate of urinary phosphorus excretion amounts to only a fraction of 1% of the rate of phosphorus excretion in the feces. Our results with respect to this relation are in agreement with the earlier observations of Forbes and his coworkers ('22). In contrast, urinary phosphorus excretion in man may exceed the excretion of fecal phosphorus (Clark, '25).

The problem of endogenous and exogenous fecal constituents has long been rceognized, particularly with respect to nitrogen. The endogenous nitrogen in feces has been estimated by *in vitro* digestion of fecal protein; a good account of this early work is given by Mayer ('25). This method was criticized by Mendel and Fine ('12), who proposed to measure the endogenous nitrogen in feces as the fecal nitrogen resulting from a protein-free diet. Mendel's method, however, is not ideal either, because it introduces an abnormal condition. This criticism also applies to Nicolaysen's ('37) attempt to measure the endogenous fecal phosphorus excretion of rats by feeding them a diet of 95% sucrose, 4% agar and 1% sodium chloride.

The use of P^{32} as a tracer for measuring endogenous fecal phosphorus has the great advantage of permitting experiments under normal conditions. Hevesy ('48) determined the endogenous fecal phosphorus in man by injecting P^{32} and using the specific activity of urinary phosphorus as an index for the specific activity of that part of plasma phosphorus which is secreted into the intestinal contents.

The trials described here appear to constitute the first attempt to estimate the endogenous fecal phosphorus in cows,

and thus measure the "true" digestibility of phosphorus in their feed and also their secretion rate of body phosphorus into the intestinal tract. Phosphorus secretion, in this report, includes all processes that transport phosphorus from the body proper to the intestinal contents. It does not distinguish among the various types of processes involved in this transport, such as diffusion, active secretion, or the sloughing off of structural elements.

METHODS

A Jersey cow weighing 427 kg (trial XII), eating daily, on the average, 10.6 ± 0.4 kg [4] air-dry food, composed of nearly equal parts of alfalfa hay and a mixture of concentrates, and yielding on the average 9.8 ± 0.2 kg milk per day, was injected twice daily with 1.3 millicuries (mc) of P^{32} contained in a nearly neutral solution isotonic with plasma. The injections were made intravenously using a plastic tube as described earlier (Ralston, Kleiber and Smith, '49). An injection lasted a fraction of a minute. The injections were continued over a period of 23 days. During this period 110 blood samples, 44 milk samples, 49 urine samples, and 31 samples of feces were taken. The cow was kept in a respiration chamber with a Ritzman urine and feces separating device (Kleiber, '35) which permitted collection of feces and urine. Unfortunately measurement of the food intake of this cow was not continued through the post-injection period.

A second Jersey cow (trial XIII) weighing 430 kg and also producing 10 kg of milk daily was similarly injected with P^{32} over a period of 10 days. Her food intake, measured over a 9-day post-injection period, amounted to 3.5 kg air-dry food per day. Table 1 summarizes this information on the cows.

The P^{32} content of the samples was measured by pipetting 0.1 or 0.2 ml of the liquids, such as plasma or a solution of fecal ash, on a circular disk of lens cleaning paper, with a 2.5-cm diameter, held on a copper planchet with a 3.1-cm

[4] The figure following the ± sign expresses the standard error of the mean.

diameter, letting the sample dry and counting the disintegration rate with a Geiger counter. The counting efficiency of the instrument, $\frac{\text{counts per minute}}{\text{disintegrations per minute}}$ was determined by making suitable dilutions of the injected solution ($1:10^3$, $1:10^4$ and $1:10^5$), whose radioactivity was reported by the Oak Ridge National Laboratories. Using such frequently measured counting efficiencies (usually about 27%), the counts per minute were calculated to disintegrations per minute and the latter to microcuries of radioactivity (1 microcurie = 2.22 million disintegrations per minute). Special counts with various amounts of shellac or milk added to the planchet indicated that the effect of self-absorption of P^{32} radiation in our samples could be neglected. Coincidence effects had only small influence in the counting range used and could be eliminated by using counting efficiencies obtained with dilutions of standards that had approximately the activity level of the samples. The results are expressed as specific activity, microcuries P^{32} per gram phosphorus, and were calculated to the first day of injection using the half-life of P^{32} of 14.3 days.

TABLE 1

General information

TRIAL	WT. OF COWS	MEAN INTAKE OF AIR-DRY FEED	DAILY MILK YIELD	DURATION OF INJECTION PERIOD	PERIOD DURING WHICH ENDOGENOUS PHOSPHORUS IN FECES WAS CALCULATED
	kg	*kg*	*kg*	*days*	
XIIa	427	10.6	9.8	23	13th to 23rd day of injection
XIIb	427	?	9.8	23	3rd to 10th day after last injection (milk) 5th to 12th day after last injection (feces)
XIII	430	3.5	9.8	10	3rd to 7th day after last injection (plasma) 5th to 9th day after last injection (feces)
XVII	481	10.0	9.9	3	6th to 9th day after last injection (milk)[1] 8th to 11th day after last injection (feces)

[1] No momentary feces samples collected from the third to the 6th day.

RESULTS

From the 13th to the 23rd day of the injection period in trial XII, the specific activity in the blood plasma could be expressed as a linear function of time because the line of least square logarithmic interpolation,

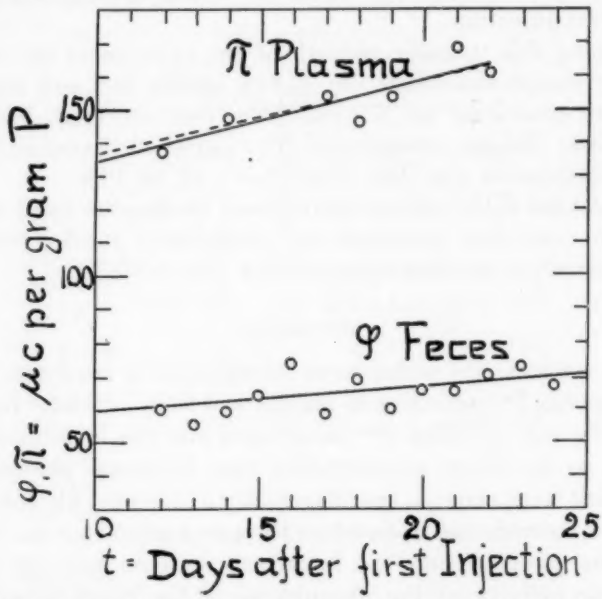

Fig. 1 Specific P^{32} activities during "steady" period, trial XII.
Regression lines: for plasma — dotted line $\log = \pi\ 2.0619 + 6.9 \times 10^{-3}$ t, full line $\pi = 110 + 2.42$ t, for feces — $\phi = 52.5 + 0.709$ t.

$$\log \pi = 2.0619 + 6.9 \times 10^{-3}\ t \qquad (1)$$

was not significantly different from the line of the linear interpolation

$$\pi = 110 + 2.24\ t. \qquad (2)$$

In both cases, π stands for the plasma specific activity in microcuries of P^{32} per gram phosphorus, and t signifies time in days after the first injection. Figure 1 shows the two regression lines and the observed values of the specific activity.

The specific activity of the feces during this period may also be expressed as a linear function of time, namely

$$\phi = 52.5 + 0.709\ t, \qquad (3)$$

where ϕ stands for the fecal specific activity in microcuries P^{32} per gram phosphorus and t, as above, for the days after the first injection.

During this "steady period" of the experiment the cow's daily phosphorus intake (in 10.4 kg alfalfa hay and concentrates) amounted to 37.1 gm. The cow excreted daily in her feces 32.5 gm phosphorus. The apparent digestibility of the phosphorus was thus $\frac{37.1 - 32.5}{37.1} = 0.12$, or 12%.

For trial XIII, with a lower food intake, the fecal phosphorus excretion exceeded the phosphorus intake, leading to a negative apparent digestibility (see table 2).

DISCUSSION

To estimate the endogenous phosphorus in the feces from the specific P^{32} activities of plasma and feces, one may formulate the rate at which P^{32} is secreted into the intestinal contents as $s\pi$, where $s =$ secretion rate in grams phosphorus per unit time, and $\pi =$ specific activity of secreted phosphorus.

This formulation is based on the assumption that the phosphorus secreted into the intestinal contents has the same specific activity as the phosphorus of the blood plasma, π. This assumption is supported by the observation that the specific P^{32} activity of the acid-soluble fraction of the plasma (trichloracetic acid filtrate) and that of the whole plasma tend to become equal three days after injection (unpublished results).

Since, furthermore, the daily increase in the plasma specific activity during the steady period amounted to only about 2% of the activity, it is reasonable to assume that during this period the specific activity among the various phosphorus compounds in the plasma was nearly equalized (compare Hevesy, '48) and was also the same in the phosphorus com-

pounds of the interstitial tissue fluid with which the blood phosphorus is in rather rapid exchange (Kleiber, Smith and Ralston, '50).

TABLE 2

Ratios of specific P^{32} activities and secretion rates of endogenous phosphorus

TRIAL	PERIOD	DAILY FOOD INTAKE		DAILY FECAL EXCRETION	RATIO OF SPECIFIC P^{32} ACTIVITIES		SECRETION RATE OF ENDOGENOUS PHOSPHORUS	
		Air-dry feed	P	P	Feces: plasma	Feces: milk	Calculated from plasma activity	Calculated from milk activity
		kg	*gm*	*gm*				
XII	13th to 23rd day of injection	10.6	37.1	32.5	0.43 ± 0.01	0.45 ± 0.01	14.0	14.6
	3rd to 12th day after last injection	...	...	49.1	..	0.35 ± 0.02	...	17.2
XIII	3rd to 9th day after last injection	3.5	12.2	14.5	0.70 ± 0.02	0.58 ± 0.02	10.1	8.4
XVII	6th to 11th day after last injection	10.0	35.0	35.2	..	0.36 ± 0.01	...	12.7

If $\bar{\pi}$ stands for the mean specific activity of plasma during a day, and s for the secretion rate of endogenous phosphorus, then the daily rate of P^{32} secretion into the gastrointestinal tract will be $s\bar{\pi}$.

Similarly, the daily rate of P^{32} excretion in feces may be expressed as $f\bar{\phi}$, where f = rate of fecal phosphorus excretion and $\bar{\phi}$ = mean specific activity of fecal phosphorus during the day.

In earlier trials, with single injections of P^{32}, we noted that the maximum specific activity in the feces occurred two to three days after injection of the P^{32} into the blood stream. We may therefore assume that the amount of P^{32} secreted into the intestinal contents during one day will, on the average, be excreted in the feces two days later. Thus we may formulate

$$s\bar{\pi}_{(t)} = f\bar{\phi}_{(t+2)} \tag{4}$$

$$\text{or } \frac{s}{f} = \frac{\bar{\phi}_{(t+2)}}{\bar{\pi}_{(t)}} \tag{5}$$

The ratio between the secretion rate of endogenous phosphorus into the tract and the excretion rate of fecal phosphorus, which in turn is equal to the ratio of endogenous to total phosphorus in the feces, is equal to the ratio of the mean specific activity of the fecal phosphorus to the mean specific activity in the plasma two days earlier.

For the period from the 13th to the 23rd day of injection in our trial XII the ratio of fecal to plasma specific activity averaged 0.43 ± 0.01, indicating that 43% of the fecal phosphorus was endogenous. The rate of secretion of endogenous phosphorus into the tract may be calculated from the rate of fecal phosphorus excretion and the ratio of endogenous to total phosphorus in the feces since, according to equation 5, $s = f \frac{\bar{\phi}_{(t+2)}}{\bar{\pi}_{(t)}}$. During the steady period of trial XII the rate of fecal phosphorus excretion was 32.5 gm per day, and the ratio: endogenous to total fecal phosphorus was 0.43; therefore the secretion rate of endogenous phosphorus $s = 32.5 \times 0.43 = 14.0$ gm endogenous phosphorus per day.

For the cow in trial XIII the rate of phosphorus excretion from the third to the 9th day after the last P^{32} injection amounted to 14.7 gm per day, of which 0.2 gm may be estimated as urinary phosphorus, leaving 14.5 gm of fecal phosphorus per day. The ratio of specific P^{32} activity in feces of a day, measured in momentary samples, to the daily mean of specific activity in plasma, collected two days earlier, averaged 0.70 ± 0.02. The cow, therefore, secreted daily

$14.5 \times 0.7 = 10.1$ gm of endogenous phosphorus into the tract. We have not yet sufficient data to decide conclusively whether the difference in the daily rate of phosphorus secretion, 14 gm in trial XII compared to 10 gm in trial XIII, shows a difference in phosphorus secretion between two cows or indicates a positive correlation between the level of food intake, or fecal phosphorus excretion, and the secretion rate of endogenous phosphorus. The latter view is favored by two additional trials in which the specific activity of the feces could be compared with that of the milk, which as a rule is similar to the specific activity of plasma, especially several days after the last injections (unpublished results; also see table 2).

A positive correlation between endogenous fecal phosphorus and food intake would indicate a similarity between the metabolic behavior of phosphorus and that of nitrogen. Endogenous nitrogen has been discussed by Mitchell ('26).

Subtracting the endogenous from the total fecal phosphorus in trial XII led to the exogenous fecal phosphorus, namely $32.5 - 14.0 = 18.5$ gm phosphorus per day. On the basis of this result the "true" digestibility of the phosphorus was calculated to be $\frac{\text{phosphorus in food} - \text{exogenous fecal phosphorus}}{\text{phosphorus in food}} = \frac{37.1 - 18.5}{37.1}$ $= \frac{18.6}{37.1} = 0.50 = 50\%$.

For trial XIII the exogenous fecal phosphorus amounted to $14.5 - 10.1 = 4.4$ gm per day and the "true" digestibility of phosphorus to

$$\frac{12.2 - 4.4}{12.2} = 0.64 = 64\%.$$

SUMMARY AND CONCLUSIONS

1. Intravenously injected radioactive phosphorus, P^{32}, used as a tracer for phosphorus exchange, permitted estimation of the endogenous phosphorus in the feces of lactating cows.

2. For a steady state, reached after 12 days of two daily injections of P^{32}, and for a period starting three days after the last injection, the ratio of endogenous to total fecal phos-

phorus could be regarded as equal to the ratio of the mean specific P^{32} activity in feces to the mean specific activity in the blood plasma of two days earlier.

3. In a cow eating 11 kg air-dry feed and producing 10 kg milk per day, 43% of the fecal phosphorus was endogenous. For another cow, producing the same amount of milk but eating only 3.5 kg of air-dry food daily, 70% of her fecal phosphorus was of endogenous origin.

4. The rate of secretion of endogenous phosphorus into the intestinal contents amounted to 14 gm per day for the cow on the larger food intake and 10 gm per day for the cow on the smaller food intake.

Comparisons with two other trials, based on specific activity in milk instead of plasma, support the conclusion that the secretion rate of endogenous phosphorus is positively correlated with the level of food intake or the rate of fecal excretion.

5. Endogenous fecal phosphorus was used to calculate the "true" digestibility of phosphorus. The cow on the higher food intake showed an apparent digestibility of phosphorus of 12%. The "true" digestibility was 50%. The cow on the low food intake excreted even more phosphorus in her feces than she consumed in feed. The "true" phosphorus digestibility, however, amounted to 64%.

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EFFECT OF ENVIRONMENT ON GROWTH AND FEED AND WATER CONSUMPTION OF CHICKENS

IV. THE EFFECT OF LIGHT ON EARLY GROWTH

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(Received for publication June 2, 1951)

The first three papers of this series (Barott and Pringle, '47, '49, '50) dealt with the effect of temperature and humidity on the growth and feed and water consumption of chickens from hatch to 32 days of age. In those papers it was noted that when the temperature was 94° to 95°F. at the start, dropped uniformly to 87° to 88°F. by the 9th day, then uniformly to 80°F. by the 18th day, and then 1°F. each day until a temperature of 66°F. was reached on the 32nd day, maximum growth and efficiency of feed utilization were obtained. Relative humidity within the range of 35 to 75% seemed to make little difference in growth.

The present paper deals with the effect of light on growth when the temperature and humidity are maintained at the optimum as shown above.

PROCEDURE

In all experiments preceding those reported in this paper, the light was supplied by one 40-watt, white, fluorescent bulb. This bulb has an input of 40 watts, of which 20.5% appears as radiation in the visible spectrum (as light), 26.5% as radiation in the infra-red portion spectrum (as heat) and 53% is lost by convection and conduction (dissipated by heating surrounding air, electrode heating, bulb warmth, and so forth).

Table 1 shows how the 18.8 watts (47% of 40 watts) which are dissipated as radiant energy are distributed over the spectrum. For comparison with daylight, the table shows the distribution of the sun's rays at Washington, D.C., in May, when the sun's altitude was 57 degrees.

The one 40-watt bulb gave an intensity of light of 12 foot candles two inches above the floor. The intensity of the light reflected from a vertical wall in front of the feed trough, with the light meter held parallel to the wall, was two foot candles at the feed trough.

TABLE 1

Comparison of radiant energy in fluorescent lamp with that of sunlight [1]

WAVE LENGTH IN ÅNGSTROM UNITS	PER CENT OF RADIANT ENERGY	
	Lamp	Sun
Under 3,800	0.5	2.6
3,800 to 5,000	8.5	14.2
5,000 to 6,000	18.0	15.2
6,000 to 7,600	16.0	21.5
Over 7,600	57.0	46.5
Total	100.0	100.0

[1] General Electric Lamp Bulletin L D-1.

The same small houses with environmental control, the same equipment, feed formula and chickens from the same source as in previous experiments were used. Thirty chicks were used for each individual experimental group.

Light and dark periods (feeding and resting periods)

The 24-hour day was divided into various combinations of light and dark periods, to determine the effect on growth of total hours of light, and also the number of light periods (these represent feeding periods) each day.

Figure 1 shows graphically the relative growth of chicks between hatch and 18 days of age, which was the period of time studied in this research, obtained with various combinations of light and dark periods.

As this study of light and dark periods extended over several months, it was necessary to devise some method of determining comparative growth over the whole investigational time period. Therefore, the growth obtained with one hour of light followed by three hours of dark was taken as 100% in each series of experiments, and the growth during the same time period in the other two test houses was taken as a percentage of this value. After all values had been computed by this method, they were transferred to the chart (fig. 1).

As is shown in the figure, the best growth was obtained with a one-hour light period followed by a three- to 4-hour dark period.

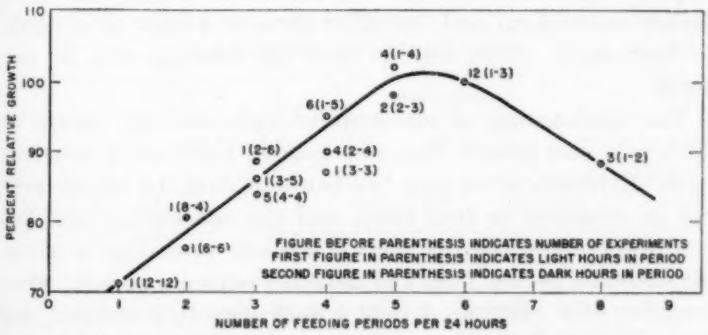

Fig. 1 Effect of duration of light and dark periods on growth of chickens.

The total feeding time (total hours of light) had no consistent effect on growth because 12 hours of light followed by 12 hours of dark gave a relative growth of 71%, while three hours of light followed by three hours of dark gave 87%. In both cases the chicks had a total of 12 hours of light available for feeding, but the growth was decidedly different.

It appears that the number of feeding periods (light periods) per day is the more important but not the only factor. For example, the combination of one hour of light and two hours of dark gave only 86% of the growth obtained with one

hour of light and 4 hours of dark, although there were 8 feeding periods in the former and only 5 in the latter combination.

Evidently if the feeding periods are too close together, the chicks do not feed fully in each period. Consequently, the feeding periods must be long enough so that the chicks can become fully fed, and the succeeeding dark period must be of such length that the crop has become empty when the lights come on again and thus the chicks are in condition to feed fully. Yet the dark period must be short enough so that the chicks do not go hungry an appreciable time before the lights come on again.

The results obtained indicate that a chick will eat all it desires in one hour and that after three or 4 hours it is ready to feed again. This routine gave the best growth, in our work.

The combination of one hour of light and two hours of dark gave less growth than one hour of light and 5 hours of dark. Evidently after only two hours of dark the chicks were not in condition to feed fully, and the succeeding light period interrupted the rest period without providing a necessary feeding period. Many of the other periods show the same tendency; for example, 4 light-4 dark; two light-6 dark; and three light-5 dark. Each gave one feeding period every 8 hours, and all gave approximately the same growth. Evidently the chicks ate all they could in the first hour of light and the rest of the light period might just as well, or perhaps better, have been dark.

In the ordinary conditions under which brooders are operated — with constant light (daylight) for from 10 to 14 hours during the 24 hours and no way to obtain the dark periods as suggested — the best thing to do is to provide light artificially during the night, so that there is light for the full 24 hours. A number of experiments in which artificial light was provided for the full 24 hours gave a growth

of approximately 90% of the best growth obtainable. Evidently the chicks, having always lived under light, will eat when they feel like it and sleep when they need to, so that they naturally provide their own rhythm of feeding and resting.

Light intensity

In a study on the intensity of lighting, the light source in each house was from a white fluorescent bulb whose characteristics are shown in table 1. To obtain the different intensities, a portion of the tube was covered until the desired intensity was obtained. To obtain the 24 foot candle intensity, a second 40-watt bulb was installed.

TABLE 2

Light intensities studied in this research

	FOOT CANDLES						
Direct	0.1	0.5	1.0	3.0	6.0	12.0	24.0
Reflected from walls	..	..	0.2	0.5	1.0	2.0	4.0

The intensities were measured: (1)) at the floor of the battery where the chicks were kept; and (2) with cell held parallel to the wall, cutting off all direct radiation from source (to obtain intensity of light reflected from walls).

The light intensities studied in this research are shown in table 2.

The growth obtained at three foot candle intensity two inches above the floor was arbitrarily taken as 100% and the growth at other intensities computed as a percentage of that. Table 3 shows the results obtained.

The results indicate that the intensity need only be great enough to allow sufficient light for feeding. The lowest intensity that furnishes enough light for efficient operation by the attendants is approximately one foot candle. Thus it

would appear that any illumination greater than one foot candle is a waste of electricity and increases operation costs. In fact, the growth obtained at the higher intensities of 12 and 24 foot candles was not equal to that obtained below those intensities. Consideration must be given to the fact that the walls were white enamel, with good reflective power.

Ultraviolet radiation

Some research has been done with ultraviolet radiation in the range between 2,800 and 3,000 Ängstrom units as a source

TABLE 3

Growth of chickens at different light intensities compared with that obtained at three foot candles

		FOOT CANDLES INTENSITY						
		0.1	0.5	1.0	3.0	6.0	12.0	24.0
					% growth			
July	1947				100		91	90
November	1949					100	91	94
December	1949				100	100	98	
January	1950			98	100	98		
February	1950		97	102	100			
April	1950	99	99	100				
Average		99	98	100	100	99	93	92

of vitamin D for chicks, but the authors know of no research where ultraviolet radiation at 2,587 Ängstrom units has been investigated to determine its effect on growth. However, a 5 years' investigation on the effect of bactericidal radiation on laying hens has been completed in this Bureau by Barott, Schoenleber and Campbell ('51), which shows an increase in the egg production of the irradiated hens over that of the controls. Five experiments were performed during this research to investigate the effect on chick growth of ultraviolet radiation at 2,587 Ängstrom units. For this purpose a WL-793 General Electric tube (a 4-watt tube radiating over 85% of its

output at 2,587 Ängstrom units) was installed in one of the houses in such a manner that the chickens were under its radiation at all times. The ultraviolet light was on continuously. All other conditions in the houses were identical.

Various intensities of ultraviolet radiation were tested, ranging from 0.5 micro-watts per square centimeter to 10 micro-watts per square centimeter. One experiment was performed at each intensity studied. For analysis of results obtained, growth in the control house was taken as 100% and

TABLE 4

Comparison of growth of chicks receiving ultraviolet radiation at 2,587 Ängstrom units with that of birds not receiving the radiation

DATE	INTENSITY OF ULTRAVIOLET RADIATION	COMPARATIVE GROWTH, CALLING CONTROLS 100%		INCREASE IN GROWTH DUE TO ULTRAVIOLET RADIATION
		With ultraviolet radiation	Controls — no ultraviolet radiation	
	micro-watts/cm²	%	%	%
Oct.–Nov. 1949	0.5	100	100	0
December 1949	1.0	104	100	4
January 1950	2.0	104	100	4
February 1950	5.0	104	100	4
April 1950	10.0	100	100	0

that in the house with ultraviolet radiation was computed as a percentage of the former. The results are shown in table 4.

As is shown in table 4, there was some indication that ultraviolet radiation at 2,587 Ängstrom units may be beneficial to the growth of chickens between time of hatch and one month of age.

Only one experiment was performed at each intensity studied, and it is evident that work must be done to determine the most beneficial intensity. No ill effects were noted and the chicks withstood the highest intensity studied, 10 micro-watts per square centimeter, without developing conjunctivitis.

Colored lights

Lights of various colors were also studied. In previous work on this subject Hammond and Titus ('41) conducted extensive tests with colored lights in rooms having walls of the same general color as the light used. Their study included

TABLE 5

Energy distribution of white, red, blue and green fluorescent lamps

COLOR	ÅNGSTROM UNITS				
	Under 3,800	3,800 to 5,000	5,000 to 6,000	6,000 to 7,600	Over 7,600
	% of radiant energy				
White	0.5	8.5	18.0	16.0	57.0
Red	0.0	0.0	1.0	10.0	89.0
Blue	1.0	30.0	9.0	0.5	59.5
Green	0.5	6.5	31.5	0.5	61.0

TABLE 6

Growth of chicks under various colored lights compared to that under red, which is taken as 100%

DATE		RED	WHITE	GREEN	BLUE
		%	%	%	%
May–June	1947	100	104		
June	1947	100	102		
Oct.–Nov.	1947	100		104	101
Nov.–Dec.	1947	100		104	102
April–May	1949	100	103	101	100
May–June	1949	100	102	100	102
June–July	1949	100	100	92	100
May	1950	100	100	92	101
Average		100	102	99	101

red, green, blue and yellow, with white for a control. Chick mortality was high because in many instances the chicks failed to eat. In experiments where comparisons were possible, no difference in growth was found that could be attributed to the color of the light.

In the present research 40-watt white, red, blue, and green fluorescent lights were used. Table 5 shows the energy distribution over the spectrum of the lamps employed.

The study of the effect of color on growth was done at two time intervals; 4 experiments from hatch to the 18th day of age were made in 1947, and 4 more experiments from hatch to the 24th day were made in 1949–1950.

Red was the only color common to all these experiments, so the growth under the other colors is expressed as a percentage of that obtained with red light.

Table 6 shows the relative growth under the various colored lights.

As all colors gave practically the same growth, it would seem that color has no effect on growth when used in a house with white enamel inside walls.

SUMMARY AND CONCLUSIONS

The effect of light on the growth of Rhode Island Red chicks during the first few weeks of age is reported in this paper.

Various combinations of light and dark periods during the 24 hours were investigated. The best growth was obtained when the lights were on one hour and off the succeding three to 4 hours in each 24 hours.

From a study of light intensity ranging from one-tenth of one foot candle to 24 foot candles, it appears that light only great enough for the chicks to see to feed is as efficient as greater intensities. An intensity of one foot candle is sufficient, and it appears that a greater intensity is simply a waste of energy and expense.

On the basis of a series of 5 experiments it appears that ultraviolet radiation at 2,587 Ångstrom units may accelerate growth.

Lights of different colors (white, red, blue and green) gave relatively the same growth. The color of the light does not seem to be important to growth.

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REPRODUCTIVE FAILURE OF RATS ON GLYCERYL TRILAURATE-CONTAINING DIETS AND ITS PREVENTION BY CERTAIN NATURAL FATS [1]

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(Received for publication May 25, 1951)

During the past several years many investigators have reported work on fat-free and low-fat diets and their relation to reproduction and viability of the young rat. Some of these investigators have suggested that fat per se is necessary for the ability of the does to rear their young, but this observation has not been confirmed by all workers. An excellent review of this work has been published by Russell ('48).

Evans et al. ('34) found that the addition of 40 mg linoleic acid and 10 mg oleic acid per rat to a fat-free diet during gestation and three times these amounts during parturition improved reproduction but did not measurably aid lactation. The inclusion of 25% lard or butterfat, however, brought the lactation performance up to that of rats on a stock diet.

Mackenzie et al. ('39) reported poor lactation in rats fed a fat-free diet supplemented with 25 mg of ethyl linoleate per rat per day. Loosli, Lingenfelter, Thomas and Maynard ('44) have also reported more rapid growth in rats suckled by mothers fed a diet containing fat (corn oil) than in rats whose mothers received a fat-free or hydrogenated coconut oil-containing diet plus methyl linoleate. This would tend to indicate that coconut oil may be deficient in a new required factor provided by corn oil.

[1] Presented before the Division of Biological Chemistry of the American Chemical Society at the Cleveland meeting, April, 1951; see abstract, p. 36C.

Deuel et al. ('47) have reported that rats maintained from weaning on a diet containing 29.4% fat showed successful pregnancies irrespective of whether the fat source was butter, margarine, or oils such as corn, cottonseed, olive, peanut, or soybean.

Bosshardt et al. ('50) have also reported that the effect of fat in the diet of mice may be due not only to the essential fatty acids present but to the fact that fat in itself may be an essential nutrient.

Recently Kaunitz and Slanetz ('50a, b, c) have reported the possible existence of a factor in lard, possibly related to vitamin A, that seems to have an effect upon reproduction and lactation in the rat.

Henderson et al. ('45) have studied the effect of synthetic fat diets on growth and report that there was no signfiicant difference between milk fat and triolein diets.

Branion et al. ('47) quote unpublished data from their laboratory showing that the hatchability of eggs laid by birds reared on a semi-purified diet was increased from 7.6% to 49.8% by the addition of 10 parts of wheat germ, replacing the same amount of polished rice. They state, ". . . We were by no means satisfied that this increase was due to vitamin E alone, if at all." The use of wheat germ oil in reproduction experiments was investigated earlier by Vogt-Möller ('39), while Juhász-Schäffer ('31) found that the addition of wheat germ oil to embryonic tissue cultures greatly increased the amount of growth.

Since 1948 we have been interested in the viability of young rats produced from does on synthetic fat diets. In this paper we are presenting evidence suggesting that a hitherto unidentified factor present in natural fats may be necessary for the successful rearing of these young.

EXPERIMENTAL

Weanling rats of the Sprague-Dawley strain were used. The males were placed on a commercial stock diet, and the

females were individually caged and placed on the experimental diets.

At 10 weeks of age each female was mated by placing a male in the cage with her for a period of 4 to 6 days.

Toward the end of the gestation period the does were carefully observed, and as soon as the pups were born they were examined and counted. (On the unsupplemented diets an accurate count was not always possible, due to the fact that the doe would often eat each pup as soon as it was born.)

The litters were observed daily in order to note the length of survival of the pups. The survival time is reported, in cases where all pups were eaten or died, as the length of time from birth until the last pup in the litter died.

The pups were handled as little as possible during the suckling period. Three weeks after birth the surviving pups were again counted, weighed and the number of survivors and weight of the litter recorded.

During the course of each experiment the females were observed closely for signs of any deficiency.

Experiment I

In the first experiment a relatively purified natural oil (corn oil) was compared with wheat germ oil.[2] This experiment was continued over three generations in order to attempt to deplete the rats of any factor which might be present in wheat germ oil and not in refined corn oil. In the first generation, 8 weanling rats were placed on each of the diets given in table 1.

The females were mated, as described, after being on diet for 8 to 10 weeks. Each female was remated until she had borne three litters. In a few cases this was not accomplished, due to the development of respiratory diseases or tumors.

During this experiment total weight gains and food consumption of each rat from weanling age until mating age were measured. However, no significant differences were found.

[2] Viobin, prepared by ethylene dichloride extraction.

Ten female rats taken from the third litters of rats on the corn oil diet and 10 females from third litters of rats on the wheat germ oil diet (these are the second generation rats, table 2) were placed on the same diets as their dams. These females were mated as before with males receiving the commercial stock diet. The females were remated again until each had borne at least three litters, if possible. There was considerable difficulty in obtaining third litters on both diets

TABLE 1

Composition of diets used in experiment I

INGREDIENT	CORN OIL DIET	WHEAT GERM OIL DIET
Vitamin-free casein (%)	24	24
Sucrose (%)	69.5	69.5
Salts 446[1] (%)	4	4
B vitamin premix[2] in cerelose (%)	0.2	0.2
Choline dry mix (%)	0.4	0.4
Wheat germ oil (%)	..	2.5
Corn oil[3] (%)	2.5	..
Dl-alpha-tocopherol acetate	12 mg/100 gm diet	..

Vitamins A and D were supplied as cod liver oil by dropper once a week.

[1] Spector ('48).

[2] Vitamin mix: thiamine 500 mg, riboflavin 600 mg, pyridoxine 300 mg, calcium pantothenate 3 gm, niacin 2 gm, folic acid 100 mg, 2-methyl-1,4-naphthoquinone 100 mg, vitamin B_{12} 5 mg, biotin 10 mg, cerelose to make up to 500 gm.

[3] The corn oil used was Mazola, purchased locally.

due to a high incidence of respiratory disease. Included in both diets was a supplement of 2% animal protein factor (APF).[3] This material had no effect on viability, the litter survival being 60% for the corn oil diet and 85% for the wheat germ oil diet as in the first generation (see table 2).

Ten females were again taken from the third litters of rats on each of the above diets (third generation, table 2) and were again placed on experiment. For this generation, a diet containing a synthetic fat (glyceryl trilaurate) was used in place of the corn oil diet to ascertain whether the

[3] Lederle.

females could raise young to weaning age without the presence of any natural fat in the ration. The 10 females from the rats receiving the corn oil diet were placed on a similar diet with 2.5% glyceryl trilaurate as the fat supplemented with 0.1% methyl linoleate to provide essential fatty acids and 0.0025% alpha-tocopherol as the source of vitamin E. The 10 females from the rats receiving the wheat germ oil diet were placed on the same diet but with a supplement of 2% glyceryl trilaurate and 0.5% wheat germ oil.

The rats grew well on these diets and showed no symptoms of any deficiency. They were mated as before, but all the rats on both diets failed to raise any of their litters to weaning age. The rats appeared to conceive and reproduce normally and the pups appeared normal at birth. However, within three to 5 days after birth all pups in all litters either died or were eaten by the dams. Therefore, the wheat germ oil was increased to 2.5%, replacing the glyceryl trilaurate, and in order to keep the vitamin E the same in both diets, the alpha-tocopherol level in the 2.5% glyceryl trilaurate diet was increased to 0.0125%.

The females which received the 2.5% wheat germ oil (WGO) diet were mated three weeks after the change in diet was made. They produced litters which showed 100% litter survival, while there was still 0% survival of pups from does on the synthetic fat diet, indicating that the vitamin E level was not the limiting factor and that it was possible to cure female rats after they had lost a litter.

An attempt was made to cure 5 of the rats that had had three unsuccessful litters. However, only two females (8 matings) became pregnant, and in each case the litters failed to survive. This failure may possibly be attributed to the depletion of the rats beyond the possibility of recovery. The results of the experiments with three generations are summarized in table 2.

Experiment II

In a second experiment the fat level in the diet was increased to try and improve the survival percentage. First,

4 weanling rats were placed on a 10% glyceryl trilaurate diet to determine whether this fat at that level was toxic. This was done since Stetten et al. (Stetten and Salcedo, '45; Keston et al., '45) had found ethyl laurate to be toxic when fed to rats on low-choline diets. These rats grew very well and seemed perfectly normal at 4 weeks, so it appeared safe to use this level of this fat in further reproduction studies. Since this is a steroid-free diet, the effect on reproduction of the addition of certain known steroids was studied. Cholesterol,

TABLE 2

Summary of experiment I

GENERATION	NO. OF RATS PER DIET	LITTERS			LITTERS		
		Born	Weaned		Born	Weaned	
		no.	no.	%	no.	no.	%
		2.5% Corn oil			*2.5% Wheat germ oil*		
1	8	20	12	60	24	20	83
		2.5% Corn oil			*2.5% Wheat germ oil*		
2	10	35	21	60	28	24	85
		2.5% Glyceryl trilaurate			*2.5% Glyceryl trilaurate + 0.5% WGO* [1]		
3	10	27	0	0	8	0	0
					2.5% Wheat germ oil		
					15	15	100

[1] Wheat germ oil.

being a known animal sterol, was used to supplement one group of rats and progesterone plus stilbestrol were added to the diet of another group. These were tried on the basis of the report of Levin et al. ('50) that wheat germ oil contains estrogenic and progestational activities. Ten weanling female rats were placed on each of the three diets, the composition of which is given in table 3.

The rats on the high-fat and the high-fat plus cholesterol diets grew normally, but due to low food consumption those receiving the hormone supplements showed retarded growth and could not be mated until approximately two weeks after

the others. The matings were carried out as before, except that the hormone supplement was discontinued during the time the males were in the cage with the females. The litter survival on all three diets was 0%.

TABLE 3

Composition of diets used in experiment II

INGREDIENTS	DIETS		
	1	2	3
Casein (%)	24	24	24
Sucrose (%)	61	61	61
Salts 446[1] (%)	4	4	4
Glyceryl trilaurate[2] (%)	10	10	10
A and D concentrate (100,000 A/gm, 10,000 D/gm)	10 mg/100 gm diet	10 mg/100 gm diet	10 mg/100 gm diet
Methyl linoleate	100 mg/100 gm diet	100 mg/100 gm diet	100 mg/100 gm diet
Alpha-tocopherol acetate	12.5 mg/100 gm diet	12.5 mg/100 gm diet	12.5 mg/100 gm diet
Choline dry mix (%)	0.4	0.4	0.4
B vitamin mix[3] (%)	0.5	0.5	0.5
Cholesterol	..	100 mg/100 gm diet	..
Progesterone	..	..	200 μg/100 gm diet
Stilbestrol[4]	..	..	200 μg/100 gm diet

[1] Spector ('48).

[2] The glyceryl trilaurate was obtained from the El Dorado Oil Works, San Francisco, California.

[3] See footnote 2, table 1.

[4] For the first 4½ weeks progesterone alone was fed; then stilbestrol was added to the diet, whereupon the rats refused to eat. Not until the diet was diluted to contain 10 μg progesterone and 10 μg stilbestrol/100 gm diet did the animals resume eating normally.

It was then decided to split groups 1 and 2 into two subgroups. Five females from each group, after having had a litter which died, were transferred to a wheat germ oil diet

(group 1a). This was the same diet they had been receiving except that the 10% glyceryl trilaurate was replaced by 7.5% glyceryl trilaurate and 2.5% wheat germ oil. This was done in order to examine further whether the deficiency could be cured with wheat germ oil. Matings were continued until all

TABLE 4

Summary of experiment II

DIET	NO. OF RATS	LITTERS		DIET	NO. OF RATS	LITTERS	
		Born	Weaned			Born	Weaned
		no.	no. %			no.	no. %
Group 1				*Group 1a*			
10% Glyceryl trilaurate (GTL) + α-tocopherol acetate + methyl linoleate	10	7	0 0	7.5% GTL + 2.5% WGO[1]	4	4	4 100
				Group 1b			
				Same as group 1	5	7	0 0
Group 2				*Group 2a*			
Same as 1 + cholesterol	10	7	0 0	7.5% GTL + 2.5% WGO[1]	5	4	4 100
				Group 2b			
				Same as group 2	5	4	0 0
Group 3							
Same as group 1 + progesterone + stilbestrol	10	11	0 0				

[1] Wheat germ oil.

rats had borne one litter. Group 2a in each case continued on the same diet and served as negative controls. On both curative diets the litter survival was 100%, while the negative control groups were unable to raise any litter to weaning age. The results of this experiment are given in table 4.

Experiment III

Since from previous work it appeared that neither increasing the level of fat or vitamin E nor the addition of choles-

terol or stilbestrol and progesterone to the diet improved the litter survival of rats on the synthetic fat diet, a new series of experiments was begun.

Forty-five female weanling rats were divided into three groups of 15 rats each. Group I received a diet similar to that previously used, with the glyceryl trilaurate level at 5%

TABLE 5

Composition of diets used in experiment III

INGREDIENTS	GROUP I	GROUP II	GROUP III
Casein (%)	24.00	24.00	24.00
Salt mix 446 [1] (%)	5.00	5.00	5.00
Glyceryl trilaurate [2] (%)	5.00	5.00	2.50
Methyl linoleate (%)	1.00	1.00	..
Vitamin mix [3] (%)	0.50	0.50	0.50
Choline (%)	0.20	0.20	0.20
Sucrose (%)	64.30	64.30	65.30
Vitamin A	2,000 I.U./100 gm	2,000 I.U./100 gm	2,000 I.U./100 gm
Vitamin D	200 U.S.P./100 gm	200 U.S.P./100 gm	200 U.S.P./100 gm
Vitamin E	15 mg/100 gm	15 mg/100 gm	..
Wheat germ oil (%)	..	..	2.5
Ketonic fraction of wheat germ oil	..	≏ 10% WGO [4]	..

[1] Spector ('48).

[2] The glyceryl trilaurate was obtained from the El Dorado Oil Works, San Francisco, California.

[3] See footnote 2, table 1.

[4] WGO, wheat germ oil.

and the methyl linoleate supplement increased from 0.1% to 1% of the diet. This was done to be certain that the essential fatty acid content of the diet was adequate.

Group II were placed on the same diet supplemented with a ketonic fraction of wheat germ oil equivalent to 10% of the oil, prepared according to the method of Girard and Sandulesco ('36). This was done in an attempt to concentrate the active fraction of the oil. Group III were used as positive controls and received 2.5% glyceryl trilaurate and

2.5% wheat germ oil as the source of fat. The composition of the diets is given in table 5. When the females were 8 to 10 weeks of age, they were mated as previously described. The results are given in table 6. It appears from this experiment that the Girard ketonic fraction of wheat germ oil is not a concentrate of the factor (or factors) necessary for the survival of young rats.

TABLE 6

Summary of experiment III

DIET	NO. LITTERS BORN	% LITTERS SURVIVED
Glyceryl trilaurate (1% methyl linoleate)	5	0
Glyceryl trilaurate (0.1% methyl linoleate)	6	0
Total	11	0
Glyceryl trilaurate + ketonic fraction (1% methyl linoleate)	3	33.3
Glyceryl trilaurate + ketonic fraction (0.1% methyl linoleate)	9	44.4
Total	12	41.1
Wheat germ oil	12	83.3

DISCUSSION

When the three fats, glyceryl trilaurate, corn oil, and wheat germ oil, are compared, considering all experiments, the number of litters born was 108, 55 and 87 and the per cent of litters that survived was 0, 60 and 88.5, respectively.

From the data presented it can be seen that a diet containing all known dietary essentials but employing a synthetic fat, glyceryl trilaurate, as the fat source is not adequate to permit female rats to rear their young to weaning age.

The rats receiving this diet appear to grow well and show no gross symptoms of any deficiency. They appear to conceive normally, although they may carry the pups one or two days past normal gestation time. The pups, when born, usually appear normal and have no physical deformities. They appear to suckle normally. In some instances the pups grow for one or two days and their abdomens appear distended,

giving the appearance that they are receiving milk. However, in most cases the pups die or are eaten within the first three days after birth. In a few litters one or two of the pups lived as long as 9 days. Increasing the level of fat in the diet does not increase the survival rate, nor does increasing the level of methyl linoleate. The addition of cholesterol or stilbestrol and progesterone to the diet also has no effect.

The only young which have survived on these diets have been those from does which received a natural fat source or a fraction of natural fats. Wheat germ oil gave better survival than corn oil, which is more purified.

SUMMARY

Female rats fed a diet containing glyceryl trilaurate plus methyl linoleate as a fat source were unable to rear their young to weaning age.

However, when this diet was supplemented with 2.5% wheat germ oil the young were raised successfully.

It appears that there is, in wheat germ oil and other natural fats (corn oil), a hitherto unidentified factor (or factors) not identical with any of the known vitamins or essential fatty acids, which is necessary to permit the female rat to rear her young.

ACKNOWLEDGMENTS

We wish to thank Mr. Ezra Levin of the VioBin Corporation, Monticello, Illinois, for his suggestion that wheat germ oil be studied as a source of unidentified factors which may be necessary in reproduction, for a supply of wheat germ oil, and for partial support of this work. We would also like to thank Dr. J. F. Burns and Mr. V. K. Collins for the preparation of the Girard ketonic fraction of wheat germ oil, and Jean Curtiss, John Curtiss, Bernice Weiss, Charles Plapp and Sadie W. Taylor for assistance in the feeding and care of the animals during the two and one-half year period of this study.

We would also like to acknowledge the generosity of Merck and Company, Rahway, New Jersey, in supplying most of

the B vitamins and the synthetic dl-alpha-tocopherol acetate, and of the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, in supplying the folic acid and APF concentrate used in this study.

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GROWTH-PROMOTING EFFECT OF AUREOMYCIN ON YOUNG CALVES WEANED FROM MILK AT AN EARLY AGE[1]

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ONE FIGURE

(Received for publication May 28, 1951)

Numerous studies have been made of the effect of small amounts of antibiotic supplements in the diet of nonruminant animals, especially poultry and swine. In general, increased growth, better feed utilization and fewer digestive disturbances have been reported for aureomycin and other antibiotics (penicillin, streptomycin and terramycin) with growing birds (Stokstad and Jukes, '50; Whitehill et al., '50; Groschke and Evans, '50; McGinnis, '50; and others) and pigs (Jukes et al., '50; Luecke et al., '50a, b; Brown and Luther, '50; and others).

In ruminants, evidence is accumulating that aureomycin supplements, at low levels, cause an increase in the growth rate of young calves. In a preliminary report, Bartley et al. ('50) claimed that aureomycin enhanced the growth of calves from birth to 7 weeks of age by preventing scours. The animals were fed an aureomycin supplement (15 mg aureomycin per 100 lb. of body weight) daily by capsule along with a normal diet. Loosli and Wallace ('50) have also reported that

[1] This work was supported in part by a grant from the Lederle Laboratories, American Cyanimid Company, Pearl River, New York.

aureomycin in a diet of various milk substitutes, with grain and hay, stimulated the growth of calves from two to 8 weeks of age by 20%. The calves were fed a 2.8% level of aureomycin supplement, or 0.5 gm of pure aureomycin per 100 lb. of dry matter intake. These workers stated that the antibiotic reduced the severity and incidence of scours in the calves. Rusoff ('50, '51) reported that an aureomycin supplement [2] fed at a 2% level (90 to 180 mg aureomycin daily) in a simple grain ration stimulated the growth of ruminating dairy calves (14 weeks of age) by approximately 35% for the first 6 weeks. This increased growth rate declined during the 8th week, and after the 20-week experimental period (34 weeks of age) the supplemented calves showed gains similar to those of the control calves. The antibiotic-fed calves showed a better appearance and condition than the controls throughout the experimental period.

In contrast to the preceding reports, Williams and Knodt ('51) did not obtain any improvement in growth rate when a 2.2% aureomycin supplement in a dry milk substitute was fed to Holstein calves to 7 weeks of age. Adverse effects of feeding antibiotics to ruminants have been claimed in two preliminary reports. Using mature steers in a balance trial involving urea, Bell et al. ('50) obtained anorexia and diarrhea within 72 hours when 600 mg of crystalline auroeomycin were fed daily. Mild symptoms occurred when the level was reduced to 200 mg. Colby et al. ('50) also obtained adverse effects in feeding aureomycin, penicillin, and streptomycin (100 mg level daily) to growing lambs. The detrimental effects of antibiotics reported by the above investigators may possibly be explained by a species difference, by the type of ration fed, by the percentage of protein in the ration, and by the high daily level of antibiotic fed in the case of the steers (Rusoff, '51).

The mode of action of aureomycin on the growth of calves is unknown. It has been suggested that the rumen microflora

[2] Aurofac.

may be altered by the antibiotic. Pounden and Hibbs ('48) and Conrad et al. ('50) have reported that protozoa and certain morphological groups of bacteria in the rumen of young calves are associated with the type of feed being ingested. Their work indicated that the calves in which these microorganisms were present had a better appearance than those calves in which the microorganisms were delayed in becoming established. Although they did not claim that these microorganisms were involved with the utilization of the feed, they did suggest that their presence was indicative of a normal flora. In the present investigation it was thought that if these microorganisms by their presence were indicative of the normal flora of the rumen, they might show some change in numbers or types as a result of the feeding of aureomycin.

This investigation was designed to study the effect of an aureomycin supplement and crystalline aureomycin in an all-grain starter ration for young calves through 16 weeks of age, when the animals were weaned from milk at an early age. The microscopic appearance of the rumen flora of the calves receiving aureomycin was also studied.

EXPERIMENTAL

Three comparable groups of 8 purebred male calves (4 Jerseys and 4 Holsteins) each were used. The calves were obtained from the Louisiana State University dairy herd and had received colostrum for the first three days after birth. On the 4th day of age the animals were placed in individual pens with wood shavings. Up to 30 days of age they were fed whole milk daily from a nipple pail at a rate of 1 lb. per 10 lb. of body weight. Calf starter rations were fed ad libitum from the 8th day of age until a maximum of 6 lb. was being consumed daily by the Jerseys and 7 lb. by the Holsteins. A medium grade lespedeza hay was fed, beginning on the 8th day, and 120,000 I.U. of vitamin A (shark liver oil) were fed each calf daily after weaning from milk. The experimental period lasted through 16 weeks.

The percentage composition of the basal vegetable protein calf starter was as follows: cottonseed meal 35, soybean meal 5.5, yellow corn meal 31.5, rolled oats 15, wheat bran 10, steamed bonemeal 2, and salt 1.0. This calf starter contained 19.45% digestible protein, 72.02% total digestible nutrients and 5.52% crude fiber. Group I received the basal starter, group II received the basal starter plus 2% of an aureomycin supplement [3] (containing 2.5 mg of aureomycin per gram) and group III the basal starter plus 75 to 150 mg of crystalline aureomycin HCl, by capsule daily, beginning at 8 days of age. The calves in group III were given 75 mg of aureomycin daily for the first 10 weeks and 150 mg for the last 6 weeks so as to simulate approximately the amount of aureomycin being consumed per day by the calves in group II from their supplemented feed. All calves were weighed at weekly intervals throughout the experiment. Daily observations of the feces and physical appearance were made for each calf. Statistical analysis of the data was made according to the method of Snedecor ('46).

Bacteriological study

Rumen samples were taken, at weekly intervals, by means of a stomach tube over a period of 8 weeks during the experimental trial. The microscopic picture of the rumen contents of the calves covered the animals for periods of from one to 16 weeks of age. The samples were examined within two hours after collection by means of a hanging drop mount for protozoa and a Gram stain for bacteria. To afford some basis for comparison between samples of the stained preparations, a standardized loopful (approximately 0.01 ml) of the well-mixed rumen contents was spread over a circular area of 2 cm^2. An examination was made for the presence of the hay and grain microflora groups described by Pounden and Hibbs ('48) as well as for any additional groups that might appear. The groups of microorganisms used were as follows:

[3] See footnote 2, page 290.

Protozoa — any protozoa observed.
Hay I — large Gram-positive coccoids, pairs and tetrads, 2.8 × 2.3 μ.
Hay II — (a) Gram-positive thick rods, 3.2–5.5 μ long, 2.5 μ wide. (b) Gram-negative cigar shaped rods, 21 × 4 μ. (c) Gram-negative short rods in fours, 1 × 0.8 μ.
Grain I — thin Gram-positive rods (sometimes granular) 1.7–3.4 μ × 0.8 μ.
Grain II — Gram-negative rods resembling coliforms.
Misc. I — Gram-positive cocci, often in pairs, similar to Hay I, but smaller (0.5–1.0 μ). This group was not described by Pounden and Hibbs.

RESULTS AND DISCUSSION

The average daily gains of the calves, the feed efficiency, and the incidence of diarrhea through 16 weeks of age are summarized in table 1. The aureomycin-supplemented calves showed increased growth gains over the control animals. The combined average daily gain for all calves receiving aureomycin (groups II and III) was 1.44 lb., as compared to 1.19 lb. for the control animals. There was a slightly greater response to aureomycin supplementation in the Jersey calves as compared to the Holsteins, although this was not statistically significant. Their combined average daily gain was 25% greater than that of the Jersey controls, while the supplemented Holsteins gained 18% more than the Holstein controls. Combining the average daily gains of both Jerseys and Holsteins in each group, the aureomycin-supplemented groups showed approximately a 20% increase in growth rate over the control group through 16 weeks of age. An analysis of variance of the data showed that these differences in growth rate were significant at $P = 0.05$. Using various breeds of calves and both sexes, Loosli and Wallace ('50) also reported a 20% increase in gain in calves receiving aureomycin in milk substitute diets from two to 8 weeks of age.

The pure aureomycin and the aureomycin supplement, which contains minute amounts of vitamin B_{12}, protein and other factors, produced similar increased growth rates. Thus the response of the animals to the aureomycin supplement was due to its content of aureomycin and not of vitamin B_{12} or other ingredients. This further corroborates the finding of Rusoff and Haq ('51) that a vitamin B_{12} supplement had no effect on the growth of calves on an all-vegetable protein ration.

TABLE 1

The effect of aureomycin supplements on the average growth rate, feed efficiency, and incidence of scours in dairy calves through 16 weeks of age

GROUP AND RATION	AVERAGE DAILY GAINS			LB. OF STARTER CONSUMED PER LB. OF GAIN		AVERAGE INCIDENCE OF SCOURS (DAYS)	
	Jerseys	Holsteins	Group	Jersey	Holstein	For 16 wks.	After 30 days of age (weaning)
	lb.						
I Basal	0.99 ± 0.1 [1]	1.40 ± 0.03	1.19	2.47	2.32	2.5	2.0
II Basal + aureomycin supplement	1.21 ± 0.09	1.69 ± 0.14	1.45	2.64	2.07	2.0	0.6
III Basal + crystalline aureomycin	1.28 ± 0.08	1.61 ± 0.01	1.44	2.44	2.16	0	0

[1] Standard error of the mean, $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$

Figure 1 presents the growth curves of the various groups of calves, showing their response to aureomycin with respect to breed. The increased gain in weight due to aureomycin supplementation was noticeable at 5 weeks of age (approximately one week after weaning) and increased thereafter at a fairly comparable rate. Two calves in group II were fed the supplement through 20 weeks of age and showed a continued increased growth rate. This indicates that aureomycin exerts

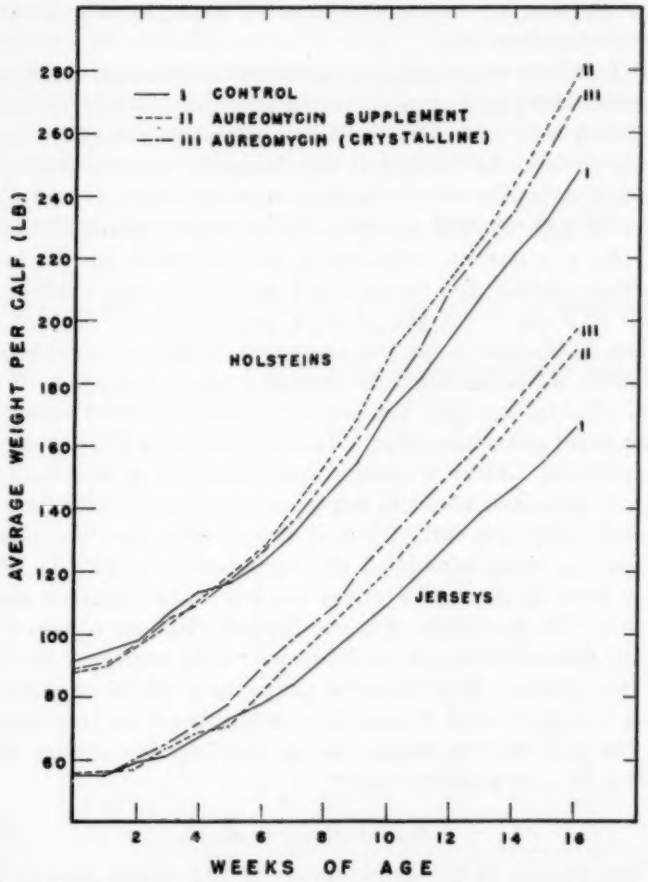

Fig. 1 Growth of calves on the basal and aureomycin-supplemented diets.

its growth stimulation on calves at a progressively increased rate.

No significant differences were observed among the various groups in regard to increased feed efficiency due to the feeding of the antibiotic (table 1). The aureomycin-fed calves consumed more of the calf starter than the control animals, in-

dicating that aureomycin stimulates appetite, which results in increased growth.

It has been reported that aureomycin enhances the growth of calves by preventing the incidence and severity of scours. In this study very little scours was encountered for the 16-week period. In table 1 it can be seen that the calves receiving capsules of crystalline aureomycin, beginning at 8 days of age, showed no evidence of scours, while the calves on the aureomycin supplement incorporated in the ration showed slightly less scours (2.0 days) than the control animals (2.5 days) for the 16-week period. At 30 days of age, group II showed a marked reduction (70%) in average days scoured, while the controls showed a slight reduction of only 20%. Up to this time the calves of group II were consuming only small quantities of calf starter containing 2% aureomycin supplement. After weaning, approximately 2 lb. of starter, which contained about 45 mg of aureomycin, were being consumed daily per calf. Thus it is probable that the level of antibiotic being consumed by the calves in group II before they were 30 days of age was too low to be effective against scours. On the basis of these limited data on scours it appears that aureomycin at the proper level prevents the onset of this disease. For practical purposes it might be suggested that a higher level of antibiotic supplement be incorporated in the calf starter ration during the first few weeks of the calf's life, to prevent scours.

Bacteriological study

The results of the examination of 122 rumen smears from 22 different calves, one to 16 weeks of age, failed to indicate any effect of the aureomycin supplement or pure aureomycin on the microscopic flora of the rumen (table 2). There was a smaller per cent of the "hay group" I and "grain group" I microorganisms found in the calves receiving the supplemented basal rations. However, the per cent of these morphological types observed in the calves receiving only the basal ration (control group) was too low to consider these

differences significant. In many instances the week-to-week variations of morphological groups in the same calf were as great as the variations of these types between the groups of calves. In some calves, the hanging drop mount would show many protozoa per microscopic field; in subsequent examination one week later the same calf might show no protozoa. In none of the calves did the protozoa appear in any number approaching that found in mature animals.

TABLE 2

The types of microorganisms in the rumen of young calves fed an aureomycin supplement or pure aureomycin

RATION	AGE GROUP	NO. OF SAMPLES	NO. OF CALVES	NUMBER OF SAMPLES WITH GROUP INDICATED PRESENT:					
				Protozoa	Hay I	Hay II	Grain I	Grain II	Misc. I
	weeks								
Basal	1–4	8	3	3	4	1	4	8	6
(no	5–8	12	4	1	2	1	7	12	10
aureo-	9–12	5	5	3	3	5	14	16	14
mycin)	13–16	5	3	2	2	3	5	5	5
Basal +									
aureo-	1–4	10	3	5	0	3	2	10	8
mycin	5–8	12	4	1	0	2	6	12	12
supple-	9–12	13	4	1	0	4	6	13	13
ment	13–16	5	3	2	0	1	1	5	5
Basal +									
crystal-	1–4	11	5	3	0	1	1	11	9
line	5–8	10	4	1	1	2	5	10	9
aureo-	9–12	14	5	4	0	6	7	14	12
mycin	13–16	5	3	3	0	2	0	5	4

If the protozoa and the microbial groups described by Pounden and Hibbs ('48) are an indication of normal rumen development, then the effect of antibiotics on the growth of young calves is apparently not due to their effect on the microorganisms in the rumen. The intestinal microflora may be altered, as has been suggested for chicks (Groschke and Evans, '50). However, any attempt to gain an adequate indication of the true nature of what is happening in the rumen

requires more than a microscopic examination of its contents. The heterogeneity of morphologically identical microbial forms makes any such examination of questionable value. The work of Gall and Huhtanen ('51) on microorganisms isolated from the rumen indicates a similarity between the types encountered by these workers and those described by Pounden and Hibbs, but wide variations may easily exist. A distinction between those microorganisms introduced by the feed into the gastrointestinal tract and those functioning in digestion is essential for determining the mode of action of antibiotics in young calves.

SUMMARY

1. An aureomycin supplement fed at a 2% level in an all-vegetable protein calf starter ration, and crystalline aureomycin fed by capsule at a level of 75 to 150 mg daily, stimulated the growth of Jersey and Holstein calves by 20% over that of control animals, through 16 weeks of age. The combined average daily gain for the supplemented calves of both breeds was 1.44 lb., as compared to 1.19 lb. for the controls.

2. Aureomycin per se was responsible for the growth-stimulating effect of the supplement and not its content of vitamin B_{12} or other factors.

3. No difference was observed in the feed efficiency as a result of feeding aureomycin. It appeared that the antibiotic stimulated appetite, which increased growth rate.

4. The pure aureomycin prevented the onset of scours (diarrhea) since it was fed at a high level (75 mg daily) early in life, while the supplement was not as effective until after 30 days of age (weaning), when higher levels of the antibiotic were being consumed.

5. A bacteriological study of the effect of aureomycin on the rumen flora of calves failed to reveal any change in the usual microscopic appearance when protozoa and the morphological groups described by Pounden and Hibbs were used as the indicator organisms.

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OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of $1000.00 established by the Nutrition Foundation, Inc., for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. Nominations for the 1952 Award, accompanied by data relative to the accomplishments of the nominee, must be sent to the Chairman of the Nominating Committee before January 1, 1952.

HENRY A. MATTILL
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CHAIRMAN, NOMINATING COMMITTEE

BORDEN AWARD IN NUTRITION

Nominations are solicited for the 1952 Award of $1000.00 and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the Jury of Award may recommend that it be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor.

The formal presentation will be made at the annual meeting of the Institute in the spring of 1952. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 1, 1952. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate consideration for the award.

HARRY J. DEUEL, JR.
University of Southern California Medical School
Los Angeles, California

CHAIRMAN, NOMINATING COMMITTEE

MEAD JOHNSON AND COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1952 Award of $1000.00 established by Mead Johnson and Company to promote researches dealing with the B-complex vitamins. The recipient of this Award will be chosen by a Jury of Award of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute in the spring of 1952.

The Award will be given to the laboratory or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year, January 1 to December 31, the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Jury of Award, nominations for this award for work published in 1951 must be in the hands of the Chairman of the Nominating Committee by January 1, 1952. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Jury of Award in its consideration of the nomination.

SAMUEL LEPKOVSKY
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